The Origin of Follicular Dendritic Cells

Dissertation

Zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Nike Kräutler

aus

Österreich

Promotionskomitee

Prof. Dr. med. Dr. sc. h.c. Adriano Aguzzi
Prof. Dr. rer. nat. Christian Münz
Prof. Dr. sc. nat. Annette Oxenius
Prof. Dr. sc. Lucas Pelkmans

Zürich 2010

Table of contents

SUMMARY

Secondary lymphoid organs (SLOs) are the sites where B and T lymphocytes encounter antigen and mount the adaptive immune response. Antigen specific B cells will first produce low affinity antibodies. Some B cells then enter the germinal center (GC) reaction, during which high affinity antibody bearing B cells are positively selected and undergo clonal expansion. An important cell type involved in the GC reaction is the follicular dendritic cell (FDC). During the GC reaction they present native antigen in form of immune complexes (ICs) to GC B cells, supplying high affinity B cells with essential survival factors, while low affinity B cells undergo apoptosis. FDCs furthermore produce Mfge8, a secreted protein needed for the removal of apoptotic GC B cells by tingible body macrophages (TBMs). FDCs have also an essential function before GCs occur – they establish T and B cell specific compartments of the SLOs, via the secretion of B cell specific chemokines and production of adhesion molecules.

FDCs, however, not only exert beneficial functions during immune responses, they also show noxious effects during pathological conditions. FDCs can trap large amounts of infectious HIV on their surface, thereby facilitating the viral spread and accelerating the course of AIDS disease. In prion diseases, FDCs are not only the major source of prion particles in SLOs, their presence is also needed for prions to invade the central nervous system (CNS).

Though much is known about the function of FDCs, little is understood about their origin. They are thought to be derived from a mesenchymal cell, as they express some fibroblast makers and show similarities to fibroblast reticular cells. However, the true founder cell remained as elusive as the question whether they are derived from a sessile or migratory cell. Development and maintenance of FDCs is based on the presence of B lymphocytes and signaling via the lymphotoxin beta receptor (LTβR) and the tumor necrosis factor receptor 1 (TNFR1).

Studying the ontogeny of FDCs I pinpointed down detailed signaling events and cell types needed for the stepwise maturation of this cell type. I revealed that putative precursors of FDCs were present at perivascular sites before follicular structures developed. While the first clustering of these cells was independent of LTβR activation, I could show that the further maturation was lymphotoxin (LT)

driven. This process was mediated by a non-B cell expressing the common cytokine receptor gamma - the lymphoid tissue inducer cell (LTi). For FDCs to undergo final maturation, B cells and TNFR1 signaling were required.

Since putative FDC precursors were first present at perivascular sites, I was interested to see whether mature FDCs are indeed derived from a cell residing along vascular structures. Pericytes are specialized cells enlacing vascular endothelial cells and were furthermore shown to be precursor cells of fibroblasts, muscle cells and fat tissue. One of the major hallmarks of pericytes is the expression of the platelet derived growth factor receptor β (PDGFRβ). To test whether FDCs are derived from pericytes, I analyzed Pdgfrb reporter mice and found that reporter expression co-localized with FDC markers. Taken together these results highly suggest a pericyte origin of FDCs and support the model that FDC like cells in vessel proximity are early FDCs.

The finding that FDCs are derived from perivascular cells expressing PDGFRβ in the spleen, probably pericytes, also gives insight into the de novo development of FDCs in non-lymphoid organs. Pericytes are a constitutive part of the vasculature in any organ. According to our hypothesis, while in future SLOs the differentiation of the local pericyte is induced by developmental cues giving ‘instructions’ e.g. through innervations of the tissue, the pericyte in the place of non-lymphoid organs usually remains ‘silent’ and does not transdifferentiate. However, the pericyte can become activated by a chronic inflammatory stimulus. The subsequent vascular remodeling results in the influx of lymphocytes and the differentiation of the pericyte into an FDC. Our model reunites the molecular and especially cellular mechanisms behind the development of the different SLOs, but also tertiary lymphoid organs (TLOs) in chronic inflammations, and is based on the presence of a cell common to all tissues, the pericyte. This insight will further help understanding the ontogeny of TLOs and the development of therapeutic strategies for chronic inflammatory and autoimmune diseases.

The second part of my thesis deals with the generation of novel transgenic methods to target several FDC related questions. Transgenic mice with FDC specific expression of genes will be used to investigate the involvement of FDCs in pathological conditions, such as prion replication, the development of autoimmunity and chronic inflammation, but also to lineage trace early FDCs in SLOs and TLOs.

Currently I am generating bacterial artificial chromosome (BAC) transgenic mice using the Mfge8 promoter to drive the expression of following genes:

- Mfge-Prnp, to restrict the prion protein to FDCs
- Mfge8-Cre and Mfge8-CreERT2, mice which will allow to conditionally ablate, label and gene target FDCs
- Mfge8-tdTomato, to fluorescently label cells actively expressing Mfge8, enabling their isolation
- Mfge8-LT, to test whether FDCs can develop in an autocrine fashion in SLOs and generate TLOs.

ZUSAMMENFASSUNG

In sekundären lymphatischen Organen (SLO) treten B und T Lymphozyten in Kontakt mit Antigen, und reagieren mit einer adaptiven Immunantwort darauf. Antigen-spezifische B Zellen produzieren als erstes Antikörper mit geringer Affinität. Einige der aktivierten B Zellen wandern in sogenannte Keimzentren, wo es zur Selektion von B Lymphozyten mit grösserer Affinität und klonalen Vermehrung dieser kommt. Eine äusserst wichtige Zelle, die an der Keimzentrumsreaktion partizipiert, ist die follikuläre dendritische Zelle (FDC). Während diesem Vorgang präsentiert die FDC natives Antigen in Form von Immunkomplexen den B Lymphozyten, wobei nur die B Zellen mit hoher Affinität für das präsentierte Antigen mit ausreichenden Überlebenssignalen versorgt werden. Die Restlichen sterben durch Apoptose. FDCs sekretieren ausserdem das Protein Mfge8, das die Beseitigung apoptotischer B Zellen während der Keimzentrumsreaktion durch tingible-body Makrophagen (TBMs) ermöglicht. Eine weitere Funktion der FDCs besteht bereits vor der Entstehung solcher Keimzentren – sie ermöglichen die örtliche Trennung der SLOs in B und T Zellareale durch die Sekretion von B zellspezifischen Chemokinen sowie die Produktion von Zelladhäsionsmolekülen.

FDCs üben allerdings nicht nur gutartige Funktionen wie zum Beispiel während der Immunantwort aus, ihre Präsenz kann auch Nachteile in pathologischen Vorgängen haben. FDCs können grosse Mengen an infektiösem HIV an der Oberfläche binden und so zur Ausbreitung des Virus beitragen. In Prionenerkrankungen, stellen FDCs nicht nur die Hauptquelle von Prionen in SLOs dar, die Invasion des Nervensystems durch Prionen geschieht nur im Beisein dieser Zelle.

Obwohl einiges über die Funktion von FDCs bekannt ist, versteht man bisher wenig über deren Entstehung. Es gibt Indizien, dass sie einen mesenchymalen Ursprung haben, exprimieren sie doch Zellmarker spezifisch für Fibroblasten und zeigen eine Ähnlichkeit mit fibroblastischen Retikulumzellen. Bisher ist weder die eigentliche Zelle, aus der die FDC hervorgeht, bekannt, noch weiss man, ob sie von einer lokalen oder migratorischen Zelle abstammt. Die Entwicklung wie auch die Erhaltung von FDCs benötigt die Präsenz von B Zellen, Zellsignalisierung via den Lymphotoxin β Rezeptor (LTβR) und Tumor Necrosis Faktor Rezeptor 1 (TNFR1).

Durch das Studium der Ontogenese von FDCs, konnte ich Zellsignalisierungsmechanismen und Zelltypen eruieren, welche die einzelnen Entwicklungsschritte dieser Zellen definierten. Es wurde deutlich, dass mögliche FDC Vorläuferzellen zuerst perivaskulär auftraten, noch bevor sich follikuläre Strukturen entwickelten. Diese erste Ansammlung von Zellen geschah unabhängig von einer Aktivierung des LTβR. Die weitere Maturation hing allerdings von Lymphotoxinen ab. Dieser Prozess wurde durch die Präsenz einer Nicht-B Zelle, welche den ´common cytokine receptor gamma´ exprimiert, ermöglicht – die ´lymphoid tissue inducer´ Zelle (LTi). Für die vollständige Entwicklung der FDCs benötigte es aber sowohl B Zellen als auch TNFR1 Aktivität.

Meinen Untersuchungen hatten ergeben, dass sich FDC Vorläufer zuerst in nächster Nähe von Gefässen befinden. Deswegen war ich daran interessiert herauszufinden, ob vollständig differenzierte FDCs tatsächlich von perivaskulären Zellen abstammen könnten. Mein Augenmerk viel dabei auf Perizyten, spezialisierte Zellen welche das Gefässendothel umgeben. Perizyten haben ausserdem Vorläuferpotential und können Fibroblasten, Muskelzellen wie auch Fettzellen bilden. Ein Kennzeichen von Perizyten ist die Expression von ´Platelet-derived growth factor β´ (PDGFRβ). Um festzustellen, ob FDCs von diesen Zellen abstammen könnten, analysierten wir Pdgfrb-Reportermäuse und stellten fest, dass das Expressionsmuster des Reporterproteins mit FDC Markern übereinstimmte. Alles in allem weisen diese Resultate sehr stark auf einen perizytischen Ursprung der FDC hin, und unterstützen das Model, in welchem FDC-ähnlichen Zellen in Gefässnähe frühe FDCs repräsentieren.

Die Erkenntnis, dass FDCs in der Milz von PDGFRβ positiven, perivaskulären Zellen - vermutlich Perizyten - abstammen, gibt auch Einblick in die *de novo* Entwicklung von FDCs in nicht-lymphatischen Organen. Perizyten sind ein fester Bestandteil eines jeden Gefässes, und deswegen in allen Organen vorhanden. Meine Hypothese besagt, dass im Falle von SLOs lokale Perizyten bereits während der Organentwicklung Schlüsselsignale – wie zum Beispiel durch die Innervierung des Gewebes – erhalten und dadurch zu FDCs differenzieren. Im Gegensatz dazu bleibt die Perizyte unter normalen Umständen in nicht-lymphatischen Organen ‚ruhend' und beginnt keine Transdifferenzierung. Im Falle einer chronischen Entzündung, wird die Perizyte aktiviert. Dies führt zu Änderungen in der Gefässstruktur und ermöglicht das

Einströmen von Lymphozyten, was in der Ausdifferenizierung der Perizyte in FDCs resultiert. Unser Modell vereinheitlicht die molekularen und zellulären Mechanismen, die der Entwicklung der verschiedenen SLOs und auch der TLOs zugrunde liegen. Laut unserem Modell basieren sie auf der Präsenz einer gemeinsamen Zelle, der Perizyte. Diese Erkenntnis wird nicht nur das Verständis bezüglich der Enstehung von TLOs erweitern, sondern auch die Entwicklung von neuen therapeutischen Strategien im Falle von chronischen Entzündungen und Autoimmunerkrankungen vorantreiben.

Der zweite Teil meiner Dissertation befasst sich mit der Entwicklung von neuen transgenen Methoden, mit welchen wir verschiedene Fragen bezüglich FDCs zu beantworten suchen. Transgene Mäuse mit einer FDC-spezifischen Expression von Genen sollen verwendet werden, um das Mitwirken von FDCs in pathologischen Zuständen, wie zum Beispiel in Prionereplikation, der Entwicklung von autoimmunen Prozessen oder chronischen Entzündungen, zu erläutern. Ausserdem erhoffen wir, dass diese transgenen Tiere uns ermöglichen, die Abstammung der entwickelten FDC von der frühen FDC zu beweisen. Ich bin ich im Prozess der Herstellung von ‚bacterial artificial chromosome' (BAC) transgenen Tieren, welche den Mfge8 Promoter zur Expression folgender Gene verwenden sollen:

- Mfge8-Prnp, um das Prionenprotein auf FDCs zu beschränken
- Mfge8-Cre und Mfge8-CreERT2 werden mir erlauben, FDCs konditionell zu entfernen, zu markieren und genetische Modifikationen auf FDCs zu beschränken
- Mfge8-tdTomato, soll das Markieren und die Isolation von Zellen, die aktive Mfge8 produzieren, erlauben
- Mfge8-LT um zu eruieren, ob FDCs in autokriner und parakriner Interaktion zur Bildung von SLOs und TLOs fähig sind.

ABBREVIATIONS

AID	activation-induced cytidine deaminase
AIDS	acquired immune deficiency syndrome
ALZ	apical light zone
ANA	anti-nuclear antibody
AP	alkaline phosphatase
APC	antigen-presenting cell
BAC	bacterial artificial chromosome
BALT	bronchial associated lymphoid tissue
BCR	B-cell receptor
BLC	B-lymphocyte chemoattractant
BM	bone marrow
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CD	cluster of differentiation
cDNA	complementary DNA
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CWD	chronic wasting disease
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
ChloR	chloramphenicol resistance gene
DC	dendritic cell
DIG	digoxygenin
dsDNA	double-strand DNA
DZ	dark zone
ER	estrogen receptor
Fc	Fc (fragment crystallizable) part of the constant domain of the antibody
FcR	Fc (fragment crystallizable) receptor
FcγR	Fc gamma receptor
FDC	follicular dendritic cell
FFI	fatal familial insomnia
FRC	fibroblast reticular cell
FSC	follicular stromal cell
GALT	gut associated lymphoid tissue
GC	germinal center
γc	common cytokine receptor gamma chain
GFAP	glial fibrillary acidic protein
GSS	Gerstmann-Sträussler-Scheinker syndrome
HE	hematoxylin and eosin
HEV	high endothelial venules
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
IC	immune complex
ICAM-1	intercellular adhesion molecule 1

IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
ISH	*in situ* hybridization
KO	knock out
LEC	lymphatic endothelial cells
LN	lymph node
LPS	lipopolysaccharide
LT	lymphotoxin
LTαβ	lymphotoxin alpha beta heterotrimer
LTβR	Lymphotoxin β receptor
LTi	lymphoid tissue inducer cell
LZ	light zone
MAdCAM	mucosal addressin cell adhesion molecule
Mfge8	Milk fat globule-EGF factor 8 protein
MS	marginal sinus
MSC	mesenchymal stem cell
MZ	marginal zone
NK	natural killer cell
NeoR	neomycin resistance gene
OVA	ovalbumin
PALS	periarteriolar lymphoid sheath
PBS	phosphate buffered saline
PK	proteinase K
pGK	Phosphoglucokinase
PP	Peyer´s patch
PrP^C	cellular isoform of the prion protein
PrP^{Sc}	pathological isoform of the prion protein
PS	phosphatidylserine
qPCR	quantitative PCR
RORγ	Retinoid-related orphan receptor gamma
RT	reverse transcriptase
SD	standard deviation
SLE	systemic lupus erythematosus
TBM	tingible body macrophage
TBS	tris buffered saline
TCR	T-cell receptor
TD	T-cell dependent
TetR	Tetracycline resistance gene
TNF	tumor necrosis factor
TSE	transmissible spongiform encephalopathy
VCAM-1	vascular cell adhesion molecule 1
WT	wild type

INTRODUCTION

THE IMMUNE SYSTEM

Overview of the immune system

As defence against microbial and viral invaders, multicellular organisms have developed a complex array of defensive devices, called the immune system. Without such effective means of protection, any living thing would soon succumb to diseases caused by foreign organisms or substances. The challenge of immunity is to discriminate foreign and altered self from non-dangerous self and to respond after recognition of a danger signal such as a pathogen with the appropriate measures (Matzinger 2002).

Plants and invertebrate animals, such as the Drosophila, have mainly developed innate responses. Foreign is recognized by pattern recognition receptors and defeated by the release of protective peptides and enzymes, which have antifungal or antibiotically properties (Zasloff 2002). The vertebrate immunity not only bases on innate, but also adaptive immune responses (Litman et al. 2005). In contrast to innate reactions, adaptive rebounds involve specific recognition of the invader, and the development of immunologic memory, which allows accelerated recognition and eradication of the same pathogen during a repeat exposure. The adaptive immune responses are carried out by lymphocytes and is divided into the humoral, or B lymphocyte mediated, and the cell-mediated, or T lymphocyte driven response. For proper responses to environmental dangers, the adaptive immune response needs to be well regulated. If the reaction is too low, we will not be efficiently protected against foreign assailants, while overreactivity of the adaptive immune response in turn may result in the attack of self. 5-7% of the human population are affected by autoreactive immune diseases.

Organs and cells of the vertebrate immune system

In adult humans, all cells needed for innate and adaptive responses are generated from the same hematopoietic stem cell (HSC), residing in the bone marrow. HSCs are pluripotent and give rise to two different stem cells, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) (Reya et al. 2001). CMPs can generate macrophages, granulocytes, but also non-immune cells such as erythrocytes and platelets. CLPs are the progenitor cells of B and T lymphocytes, the main actors of the adaptive immune response, as well as of natural killer (NK) cells and dendritic cells (DC).

Primary lymphoid organs

Primary lymphoid organs are the sites where B and T lymphocytes mature. Here, they develop from their respective precursor and acquire their functional receptors, which ascertain the antigenic specificity later needed during the immune response. The broad repertoire of these receptors (for a multitude of antigens) is mainly based on recombinase activating gene (RAG) rearrangements of the multigenes coding for B or T cell receptor (Litman et al. 2005).

B cells mature in the bone marrow (BM) in the presence of bone marrow stromal cells (BMSC). BMSC support the development of B cell precursors by direct interaction, but also by supplying them with secreted cytokines, such as interleukin 7 (IL-7). The earliest cell in the B cell lineage is the pro B cell. It expresses the B cell marker B220, as well as the Igα/Igβ heterodimers. Upon contact of the pro B cell with the BMSC, differentiation into pre B cell is induced. In contrast to the pro B cell, the pre B cell has undergone VDJ gene rearrangement of the immunoglobulin heavy chain, and in conjunction with surrogate light chains and the Igα/Igβ heterodimer, forms the pre-B cell receptor. Furthermore, pre B cells upregulate IL-7 receptor (IL-7R), allowing it to react to stromal IL-7. Further rearrangement of the light chain generates the mature B cell receptor (BCR), consisting of membrane bound immunoglobulin (mIg), in form of IgM and IgD, and the signaling transducing Igα/Igβ heterodimers. Before mature B cells leave the BM to meet antigen, they undergo negative selection, a process which leads to the elimination of autoreactive B cells (Hardy and Hayakawa 2001).

T cells develop from the CLPs in the thymus, where RAG rearrangements of the T cell receptor (TCR) multigene result in the formation of the mature TCR/CD3 (cluster of differentiation 3) complex. Mature T cells detect antigen presented via the major histocompatibility complex (MHC). Therefore, immature T cells in the thymus undergo a round of positive selection, where they are instructed by thymic epithelial cells to recognize MHC molecules. Thymocytes unable to do so do not receive essential survival signals resulting in death by apoptosis. Immature T cells furthermore pass a negative selection process, where self reactive T cells are ablated.

Secondary lymphoid organs

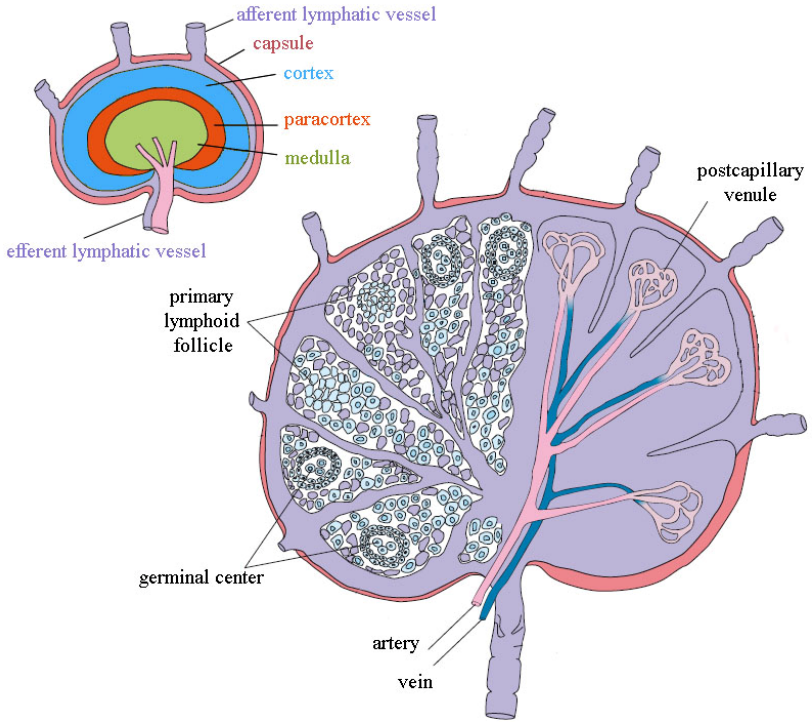


Fig. 1 Organization of the lymph node
LN is structurally divided into 3 distinct regions: cortex, paracortex and medulla (small figure). The cortex contains primary and secondary B cell follicles containing germinal centers. The paracortex is rich in T helper cells and interdigitating DCs. In the medulla plasma cells are present and lymph fluid is collected. Lymph fluid is transported to the LN via afferent lymphatic vessels and leaves the LN via the efferent lymphatic vessels. Lymphocytes migrate from the blood into the LNs emerging from postcapillary venules. Adapted from Kuby immunology fifth edition 2002

In SLOs, mature B and T cells meet the antigen. SLOs are well structured organs, containing lymphocytes, phagocytes, antigen presenting cells (APCs) and stromal cells. The purpose of SLOs is to efficiently trap and present antigen in order to mount a quick and efficient immune response. Lymph nodes (LNs), spleen, mucosal associated lymphoid tissues (MALT) and gut associated lymphoid organs (GALT), including the Peyer's Patches (PP), are SLOs. LNs and spleen are highly organized, encapsulated SLOs. While the spleen is specialized for immune responses to blood borne, thus systemic antigen, LNs collect antigen and antigen-experienced DCs from local tissues transported by lymph fluid within lymphatic vessels (Fig.1). Mammals carry a multitude of LNs present in head and neck regions, within the thorax, abdomen and limbs. The lymph from afferent vessels is collected in the subcapsular sinus, where a network of trabecula drains the LN's cortex and paracortex, until the lymph is recollected in medullary sinuses and leaves the LN via the efferent lymphatic vessel. Lymphocytes enter the LN via high endothelial venules (HEVs), specialized postcapillary venules, which allow the transmigration of lymphocytes into and out of the organ. Upon antigen exposure of the LN, antigen-specific naïve B cells become activated and migrate into primary

follicles, where they start to proliferate. Primary follicles further develop into secondary lymphoid follicles containing GCs. During the GC reaction antigen-activated B cells undergo affinity maturation of the BCR and class-switching.

The spleen is an encapsulated organ, which lies within the peritoneal cavity. In contrast to LNs, the spleen is not supplied by lymph, but filters blood-borne antigen carried into the organ by the splenic artery. Hence, the spleen raises immune responses to systemic infections. It is compartmentalized into two functionally distinct areas, the red pulp (RP) and the white pulp (WP) (Fig.2). The RP's main function is the removal of old and defective red blood cells. It consists of a network of sinusoids, vessels with a discontinuous endothelium, which allow the selection of old, less flexible erythrocytes, and contains a multitude of macrophages, removing the damaged erythrocytes. The WP is arranged around the splenic artery and arterioles. The innermost layer, the periarteriolar sheath (PALS), is rich in T cells, the B cell area is adjacent to the T cell zone and is structured in primary and secondary B cell follicles. The marginal zone (MZ) borders the RP area and contains specialized MZ B cells and macrophages. Blood-borne antigen reaches the spleen via the splenic artery, which further branches out into smaller arterioles and empties into the MZ. In the MZ antigen-antibody complexes (immune complexes) are trapped, and the early T cell independent (TI) immune response mediated by MZ B cells is induced

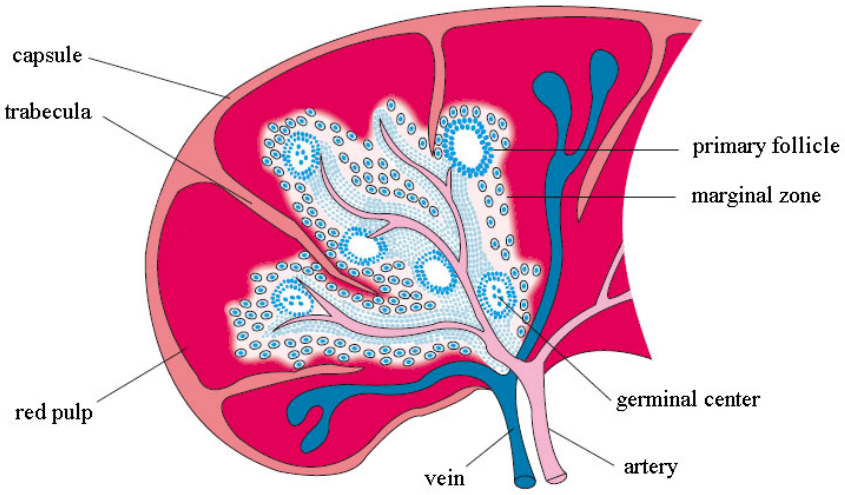


Fig. 2 Organization of the spleen
The spleen is an encapsulated organ, which lies within the peritoneum. The spleen is supplied with blood by the splenic artery, and blood leaves the organ via the splenic vein. The spleen is anatomically divided into the red pulp (RP) containing macrophages and erythrocytes and the white pulp (WP). The WP area surrounds the arterioles, and is subclassified into T cell rich, neighbouring the vessels, and a more distal B cell (follicular) area, structured in B cell follicles. At the border between WP and RP lies the MZ, consisting of specialized B cells and macrophages.
Adapted from Kuby immunology fifth edition 2002

(Zandvoort and Timens 2002). From the MZ ICs are transported into the follicle and transferred onto specialized stromal cells, the FDCs, which ensure an efficient GC generation (Ferguson et al. 2004; Cinamon et al. 2008). In MALT and GALT, antigen is not delivered by means of vessels. In the intestine for example, antigen is transported from the lumen of the digestive tract through the mucosa by specialized M cells to the underlying lamina propria, which contains the immune cells (Neutra et al. 2001).

Tertiary lymphoid organs

During persistent inflammations, either induced by a chronic exposure to a pathogen, tumor cells or during autoimmune reactions, structured lymphocytic infiltrates in non-lymphoid organs can develop, which are termed TLOs (Aloisi and Pujol-Borrell 2006). In contrast to SLOs, TLOs are neither encapsulated, nor supplied by a specialized vascular system. Similarly to SLOs, however, is the segregation into B and T cell specific zones, and the eventual emergence of B cell follicles containing GCs. A variety of target tissues of chronic inflammations are reported in humans, such as lung, liver, thyroid gland, central nervous system, gut, stomach, joints, arteries. Autoimmune diseases with reported TLO development are rheumatoid arthritis, multiple sclerosis, Hashimoto's thyroiditis, and primary biliary cyrrhosis. Other chronic inflammatory diseases are Crohn's disease and arteriosclerosis, TLOs associated with cancer occur in ductal breast carcinoma, while TLOs can also be induced by infection such as hepatitis C virus (Aloisi and Pujol-Borrell 2006).

Generation of the B cell response

Antigen naïve, mature B cells down regulate receptors for adhesion molecules expressed by BMSC, resolve the contact and leave the bone marrow. Via the blood stream they home to SLOs and TLOs, the places where they encounter antigen. Antigen binding on the mIg, results in the triggering of a signaling cascade which in turn leads to proliferation and differentiation of the mature B cells into antibody secreting plasma cells and memory B cells.

In the spleen we discriminate two kinds of mature B cells, the follicular, which constitutes the main population, and the MZ B cells (5% of the total population). The first B cells to react to antigen and differentiate into antibody secreting plasma cells are the MZ B cells, lining the marginal sinus. Their threshold to become activated is

much lower than for follicular B cells, and induction is usually TI. TI antigens frequently stimulate B cells regardless of their antigenic-specificity. For example bacterial cell-wall polysaccharides, such as lipopolysaccharide (LPS), can act as TI antigen. TI responses are usually less pronounced than T cell dependent (TD) responses, and do not result in the generation of memory or seldom in class switching. In TD reactions the stimulation of the antigen-specific B cells is mediated by direct interaction with a cognate T_H cell, a T cell responsive to the same antigen, as well as the presence of T_H cytokines. The immediate reaction to a pathogen by B lymphocytes is the generation of short lived plasma cells, which secrete low affinity antibodies as a first defensive mechanism.

The initial interaction of the B cell with the T_H cell occurs extrafollicularly in the T cell zone (Gray 1988). In a second step some of the antigen-experienced B cells migrate into B cell follicles and get involved in the GC reaction, where B cells producing higher affinity antibodies with switched Ig class are generated (Fig. 3) (Kuppers 2003). At the beginning of the GC reaction, B cells, termed centroblasts, reside in the dark zone (DZ) of the secondary follicle. They undergo several rounds of clonal expansion, during which somatic hypermutation mainly within the variable region of the Ig happens, which is mediated by the activation induced cytidine deaminase (AID). AID recognizes cytosine bases in the Ig DNA and converts it into uracil. Mismatch repair removes uracil from the DNA and error prone polymerases replace the missing DNA pairs, which results in the generation of mutations. Centroblasts expressing very little membrane-bound Ig, exit from proliferation and start expressing surface-bound Ig. These so-called centrocytes migrate to the light zone (LZ) of the germinal center, where FDC and T_H cells are located. During a process termed affinity maturation, selection of centrocytes expressing high affinity antibodies for antigen presented by FDCs as ICs, will be supplied with appropriate survival signals. At the same time centrocytes need to receive signals from cognate follicular T_H cells. Signals so far identified to be important are the interaction of T_H cells CD40L with its receptor CD40 expressed on the B cell (Guzman-Rojas et al. 2002). Low affinity B cells undergo apoptosis, start displaying eat-me signals on their surface, leading to their removal by TBMs. One of these eat-me signals is the exposure of phosphatidylserine (PS), which is usually restricted to the inner leaflet of

the plasma membrane (Fadok et al. 1992; Koopman et al. 1994; Blankenberg et al. 1998).

Cytokines secreted by the cell determine the type of class switch that occurs

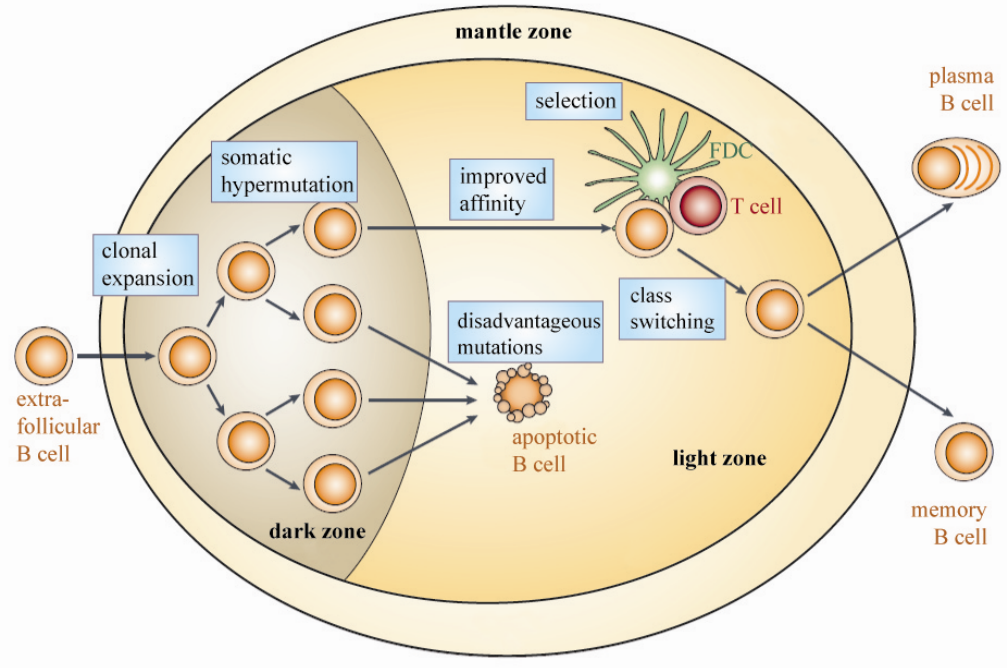


Fig. 3 The germinal center
The GC is divided into three main compartments: the mantle zone, the light zone and the dark zone. The DZ contains proliferating centroblasts, undergoing somatic hypermutation of the Ig genes. Centroblast stop dividing and differentiate into LZ centrocytes, expressing surface bound Igs. Centrocytes, with improved affinity for the antigen, are supplied with survival signals from T_H cells and FDCs. Cells with lower affinity do not receive essential signals and undergo apoptosis. GC B cells, positively selected for antigen, undergo class switching. Cytokines present decide the type of Ig to be used. B cells then undergo final differentiation into antibody secreting, short lived plasma cells or memory B cells. Adapted from Nature Reviews Immunology 2003 (Kuppers 2003)

after selection. While IL-1 promotes the generation of IgG1 and IgE, the presence of IL-4 induces a switch to IgE, IL-5 to IgA (Stavnezer et al. 2008). Before the positively selected GC B cells differentiate into plasma cells or memory B cells and leave the GC, they undergo several rounds of proliferation. Plasma cells, which are rather short lived cells, secrete high amounts of soluble Ig. Membrane bound and soluble Igs are splice variants from the very same RNA. Memory B cells on the contrary, are long lived B cells, giving rise to effector B cells very quickly after a secondary antigen exposure, without the need of a further GC reaction (Liu et al. 1991). Different cytokines and transcription factors were associated with the fate decision plasma B cell or memory B cell (Liu and Banchereau 1997), OX40, IL-3, -6,

-10 favoring plasma cell development, while CD40L and IL-4 induce differentiation into memory B cells.

FOLLICULAR DENDRITIC CELLS

Characterization of Follicular Dendritic Cells

Stromal cells constitute a major part of the lymphoid organs. In SLOs specialized reticular cells, or follicular stromal cells (FSCs), generate a network of cells, ensheathing the hematopoietic cells with their collagenous fibers and supporting the architecture of these organs by the secretion of extracellular matrix molecules.

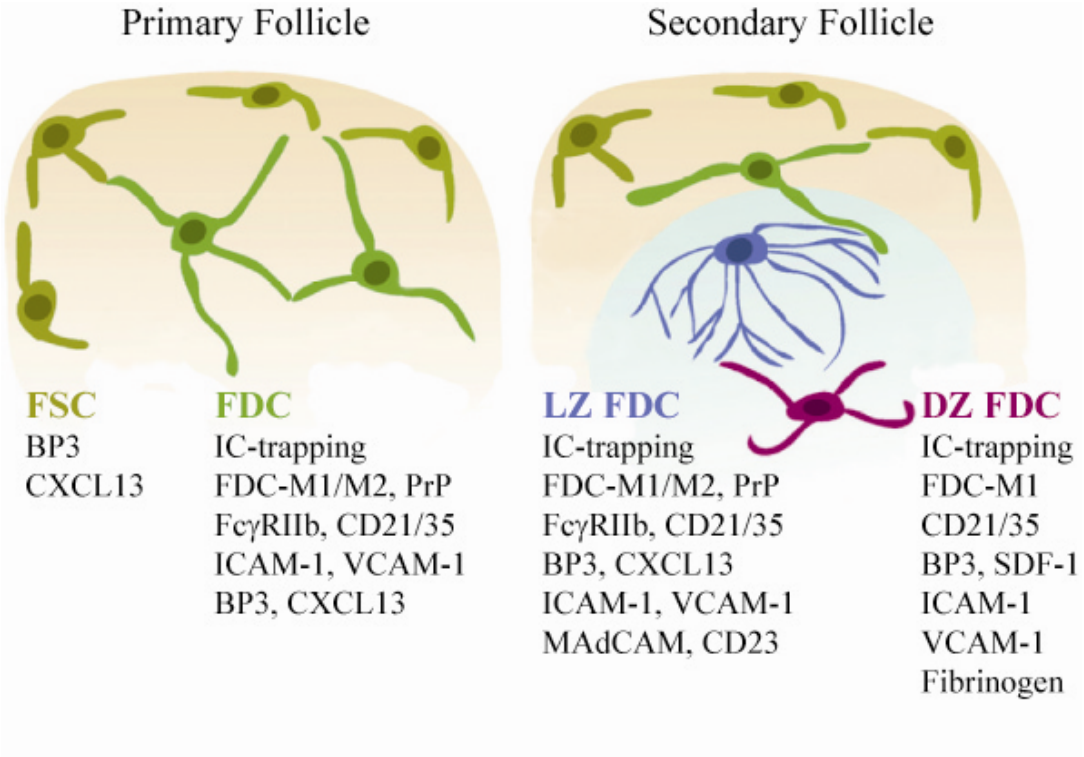


Fig. 4 Differences between FSCs and FDCs in primary and secondary follicles
In primary follicles FSCs and FDCs are present. Though FSCs express some markers shared with FDCs, B3P3 and CXCL13, they do not capture ICs. Primary follicle FDCs stain for FDC-M1 and FDC-M2, PrP, FcγRIIb, CD21/35, ICAM-1, VCAM-1. After antigen exposure, GCs are formed, leading to further differentiation of FDCs. LZ FDC express in addition MAdCAM and CD23 and have characteristic filiform dendrites, which ensheath the GC B cells. DZ FDCs lack the expression of FcγRIIb, PrP, CXCL13, and MAdCAM, but express SDF-1 and fibrinogen. Adapted from Semin Immunology 2008 Cyster

Analysis by electron microscopy revealed ultrastructural differences between the fibroblast reticular cells, and more dendritic shaped cells, termed dendritic reticular cells, which reside within GC of B cell follicles (Mitchell and Abbot 1965; Heusermann et al. 1980; Yoshida and Takaya 1989; Yoshida et al. 1993). One major reported feature of these dendritic cells, was the long-term retention of ICs, which was

dependent on the presence of complement and the Fc-part of antibodies (Papamichail et al. 1975; Klaus and Humphrey 1977; Chen et al. 1978). Subsequent studies revealed that the receptors for complement, the complement receptors, CR1 (= CD35) and CR2 (= CD21) and the Fc receptor, Fc gamma RIIb (FcγRIIb), were needed for the IC-trapping (Yoshida et al. 1993; Imal and Yamakawa 1996; Qin et al. 2000). Later these dendritic reticular cells were termed FDCs to discriminate them from the other reticular cells (Tew et al. 1982). However, transitional forms between fibroblast reticulum cells (FRCs) and FDCs were observed during GC reactions, providing evidence of their fibroblast-like, mesenchymal phenotype (Kamperdijk et al. 1978; Yoshida and Takaya 1989; Yoshida et al. 1993).

FDCs so far are determined by their ability to bind ICs. They are present in primary lymphoid follicles, but especially prominent in secondary lymphoid follicles. Here, they reside in the LZ as well as DZ of the GC, and adapt to their local functions with slightly distinct appearance and molecular characteristics (Fig. 4). Especially FDCs in GC LZ have very elaborate filiform processes forming a dense network, entangling the centrocytes. CD21 and CD35 are present on both primary and secondary FDCs. Primary follicles FDCs express BP3, FcγRIIb (CD32), vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), FDC-M1 (= Mfge8), FDC-M2 (= complement C4), CXCL13 (BLC), and the prion protein (PrP). After immunization and GC development, FDCs upregulate the listed markers and in addition start expressing mucosal addressing cell adhesion molecule (MAdCAM), CD23 (=FcεRII), which is only present in LNs, but not spleen. DZ FDCs are $BP3^{+}$ and express CD21/35, SDF-1 and fibrinogen. They show little IC-trapping, are slightly positive for FDC-M1, ICAM-1 and VCAM-1, but are negative for FDC-M2 or CXCL13, CD23 and FcγRIIb.

Function of Follicular Dendritic Cells

Secretion of chemokines/attraction of lymphocytes

One of the hallmarks of FDCs is the secretion of chemokines, which result in the recruitment of lymphocytes to the follicles of the SLOs and TLOs. One of these factors is the B lymphocyte chemoattractant (BLC, CXCL13). It is produced and secreted by FDCs as well as other sinus lining FSCs, and binds to B cells' CXCR5, thus leading to their migration into the follicles (Gunn et al. 1998). BLC is made by FDCs of the primary lymphoid follicle and LZ FDCs. DZ FDCs do not express it, but produce another chemokine, SDF-1 (CXCL12), which signals via CXCR4. SDF-1 determines the development of the GCs and is needed for the segregation into LZ and DZ. Consistent with the notion that production of BLC by FDCs strongly regulates B cell follicle and GC organization is the phenotype of mice deficient for BLC or its receptor CXCR5. These mice lack most of the LNs as well as the PPs and have disorganized GC with B-cell accumulation around the central arteriole (Ansel et al. 2000).

Production of adhesion molecules for lymphocytes

For the proper migration of lymphocytes into the organs not only chemokines, but also adhesion molecules are needed. FDCs display several adhesion molecules, which are essential for the close FDC/B cell interaction. When FDCs are isolated and co-cultured with B cells they form clusters, suggesting the presence of adhesion molecules (Tsunoda et al. 1992). Several adhesion molecules have so far been identified to be expressed by FDCs, including ICAM-1, VCAM-1, MAdCAM. ICAM-1 and VCAM-1 ligands are from the integrin family, namely LFA-1 (lymphocyte function associated antigen 1, a heterodimer of integrin αL and β2) as a receptor for ICAM-1 and the VCAM-1 receptor VLA-4 (very late antigen 4; intergrin α4β1). LFA-1 and VLA-4 are both present on B cells. Indeed, when these ligands or receptors are blocked by antibodies, *in vitro* cluster formation of FDC and B cells is severely reduced (Koopman et al. 1991).Also FSCs in the MS express ICAM-1 and VCAM-1, which is needed for the localizing MZ B cells.

Display of immune complexes and affinity maturation of B cell

One of the major features of FDCs is the trapping of ICs on their surface via the expression of Fc (fragment crystallizable) receptors, FcεRII and FcγRIIb, and complement receptors, CD21 and CD35 (Tew et al. 1982). ICs can either be composed of antigen-antibody, antigen-complement or antigen-antibody-complement. In LNs ICs are first captured by subcapsular sinus macrophages, non-cognate B cells transport the ICs into the GCs and transfer them onto FDCs, where they are retained over long periods (Phan et al. 2009). FDCs present native antigen to naïve B cells, GC B cells and memory B cells. *In vitro* it was shown that the presence of ICs is highly stimulatory to B cells and results in the upregulation of AID in the B cell, which in turn leads to somatic hypermutation of the Igs and class-switching. *In vitro* culture of GC B cells also showed that in the absence of survival factors this cell type undergoes cell death within short term. However, the addition of FDCs and T cells to GC B cell cultures lead to an increase in their survival (Burton et al. 1993). Supply with B cell specific antigen further increased their viability (Kosco et al. 1992). The exact signaling mechanisms of GC survival are not completely understood. It is believed that GC B cells are death prone by high expression of Fas receptor (FasR) as well as its ligand FasL. Unknown signals supplied by T cells and FDCs in turn lead to the upregulation of Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (cFLIP(L), which inhibits FasR-induced caspase activation (van Eijk et al. 2001). It is so far believed that in the GC B cells compete for the antigen presented by FDCs, and only B cells binding with high affinity obtain survival signals, while the remaining die and are removed by local macrophages, the tingible body macrophages (TBMs). Although, mice lacking FDCs or complement receptors, can undergo a GC reaction and class switching, this process is strongly hampered and needs the presence of high amounts of antigen as well as adjuvants (Kosco-Vilbois 2003).

Removal of apoptotic GC B cells

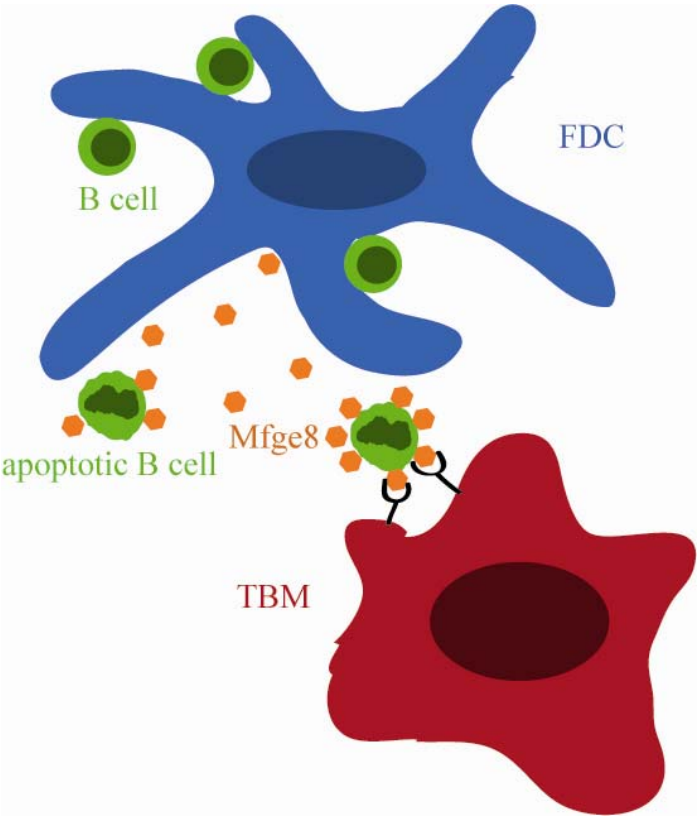


Fig. 5 Model of the removal apoptotic B cells by TBMs
FDCs supply high affinity B cells with so far unknown survival signals which inhibit the proapoptotic state. Apoptotic B cells are covered with Mfge8, secreted by the FDC, binding to the exposed PS. The RGD motif of Mfge8 leads to the recognition by TBMs integrin receptors and subsequent engulfment.

90% of the GC B cells do not survive the selection procedure and undergo apoptosis. During this process they display so-called 'eat-me signals', including phosphatidylserine (PS), on their surface and are rapidly removed by TBMs (Flemming 1885; Smith et al. 1991; Fadok et al. 1992; Koopman et al. 1994; Blankenberg et al. 1998). Under normal circumstances PS is kept on the inner leaflet to the plasma membrane by the help of flippase, during cell death the phospholipids asymmetry between inner and outer leaflet can no longer be maintained and PS becomes exposed on the plasma membrane outer surface as well. FDCs play a crucial role in the B cell removal process by secretion of milk fat globule epidermal growth factor 8 (Mfge8 = lactadherin), a PS-binding protein required for the engulfment of apoptotic cells by macrophages (Fig. 5) (Hanayama et al. 2002; Hanayama et al. 2004; Kranich et al. 2008). Mfge8 has a recognition site for PS, but also contains the integrin binding RGD (arginine-glycine-aspartate)-motif, which is recognized by integrin $\alpha v\beta 3$ expressed on TBMs, linking the apoptotic cell to the macrophage and ensuring its removal. Impairment of Mfge8-dependent removal of apoptotic GC cells has been associated with lupus-like autoimmunity (Hanayama et al. 2004).

Follicular dendritic cells during pathological conditions

Tumours with immunohistochemical characteristics of FDCs, e.g. expression of the FDC markers CD21, CD23, CD35, Clusterin do exist. FDC tumours (FDCT) are usually found in lymphoid tissues, such as LNs, tonsils and spleen, but can also be present at other sites such as gastrointestinal tract, and more recently described in lung (Denning et al. 2009).

FDCs in chronic inflammatory conditions and autoimmunity

As a constitutive part of primary and secondary follicles, FDCs can also develop during chronic inflammatory diseases to generate TLOs and stabilize their persistence. Chronic inflammations occur either during a prolonged infection with impaired clearing, as a response to a tumor or as a consequence of an autoimmune disease. Several autoimmune diseases are associated with the development of ectopic TLOs. In the thyroid gland this is Hashimoto's thyroiditis, and Grave's disease, in the joints rheumatoid arthritis, multiple sclerosis affecting the CNS, and cryptogenic fibrosing alveolitis in the lung. In the GCs of these TLOs, organ-specific and disease-associated autoantibodies are generated, by the help of the local FDCs. Infections can lead to *de novo* formation of TLOs as well, as it is the case in chronic hepatitis C, *Helicobacter pylori* induced gastritis (Aloisi and Pujol-Borrell 2006). Chronic inflammations are also associated with the development of lymphomas, which develop most probably as a consequence of continuous B cell proliferation (Hjelmstrom 2001).

Another autoimmune disease with possible FDC misfunction is systemic lupus erythematosus (SLE). Unlike organ-specific autoimmune diseases SLE affects the organism systemically. During this disease antibodies against a plethora of tissue antigens, including DNA and nuclear proteins form antigen-antibody complexes. These complexes can on one hand destroy the targeted cells, e.g. neutrophils, on the other hand lead to the activation of the complement system, but also become deposited in certain tissues, where they lead to local damage. The synovial tissue of the joint, the pleura of the lungs, the skin as well as the kidney's glomerula (glomerulonephritis) are classical places of tissue destruction in SLE. The occurrence of SLE is linked to defects in the removal of apoptotic cells by macrophages, especially the reduced clearance of GC B cells by TBMs has been reported (Baumann et al. 2002). Non-phagocytosed apoptotic cells undergo secondary necrosis, which will result in the generation of self-reactive antibodies against nuclear antigens of

these cell remnants. Supporting this theory are following observations: The absence of two factors, C1q and Mfge8, linking the apoptotic cell to the macrophage and thus enabling its removal, are associated with the development of SLE-like phenotype in mice (Walport et al. 1998; Gaipl et al. 2004; Hanayama et al. 2004; Gaipl et al. 2005). Recently, a genetic polymorphism in the human *MFGE8* gene was reported in patients with SLE (Hu et al. 2009). Surprisingly, the group of Nagata found increased levels of serum Mfge8 in patients with SLE (Yamaguchi et al. 2008).This could be explained by a dose dependent function of Mfge8. While bantam and local amounts leads to the linkage of the apoptotic cell to the macrophage, excessive Mfge8 will bind to the PS on the dying cell's surface or cover the integrin binding sites without acting as a bridging molecule. Interestingly, Mfge8 in the lymphoid follicles is produced by FDCs and mice lacking mature FDCs have impaired removal of apoptotic B cells and increased levels of autoantibodies (Chin et al. 2003; Kranich et al. 2008). This suggests a beneficial role of FDCs in the prevention of self-reactive immune diseases, by controlling the appropriate removal of B cells during the GC reaction via Mfge8.

Infectious diseases with FDC involvement

FDCs can also act as a 'Trojan horse' during certain infectious diseases. Upon infection with human immune deficiency virus (HIV) and seeding of the viral particles to lymphoid organs, FDCs represent the major reservoir of HIV, trapping them on their surface (Racz 1988; Pantaleo et al. 1993; Cavert et al. 1997). The particles bound as ICs not only help initiating and maintaining the HIV-specific immune response, but can also act as a constant source of infection for nearby $CD4^+$

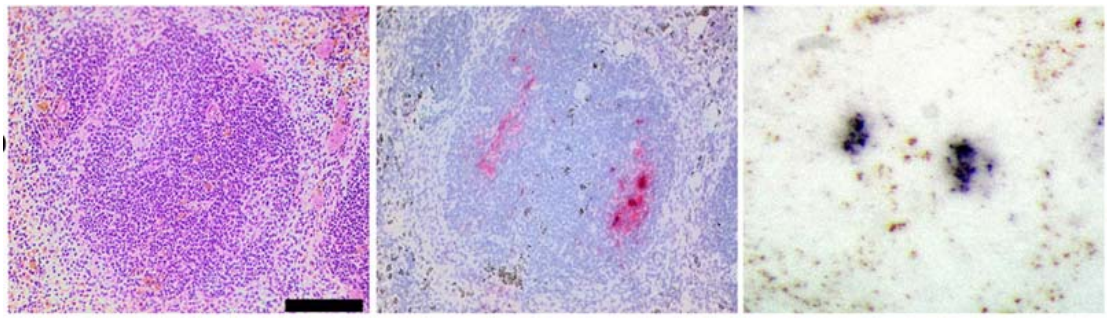


Fig. 6 Colocalization of FDCs with PrP^{Sc} deposits
Consecutive splenic cryosections stained with hematoxilin-eosin (HE) (left), or with the FDC marker FDC-M1 (middle) and a histoblot to detect PK resistant PrP^{Sc} (right). Adapted from J Neuroim (Heikenwalder 2007)

T-cells, the main target of HIV (Schrager and Fauci 1995). Surprisingly, despite being bound as ICs and hence covered with antibodies, virions are still infectious for (Heath et al. 1995). Hand in hand with the very efficient accumulation of retroviral virions, FDCs rapidly lose their ability to efficiently trap and retain antigens, apart from viral ones, as shown by Masuda *et al.* using a murine model of HIV (Masuda et al. 1993; Masuda et al. 1995). During further progression of disease, the lymphatic microenvironment, including the FDC networks, is eventually destroyed, a phenomenon that also occurs during other viral or bacterial infections (Pantaleo et al. 1993; Smelt et al. 1997). Surprisingly, FDCs were also shown to be involved in a class of diseases affecting the CNS, the transmissible spongiform encephalopathies (TSEs). TSEs are fatal neurodegenerative disorders, which can occur in many different mammalian species. TSEs occurring in humans are Kuru, Gerstmann-Sträussler-Scheinkler syndrome (GSS), Creutzfeldt-Jakobs disease (CJD), new variant CJD, and fatal familiar insomnia (FFI), in cattle as bovine spongiform encephalopathy (BSE) or 'mad cow disease', scrapie in sheep and goat as well as chronic wasting disease (CWD) in elk and deer. TSE's common feature is the deposition of large extracellular amyloid-like aggregates, accompanied by neuronal loss and spongiform changes in the brain, gliosis (Prusiner et al. 1983; Weissmann 2004). TSEs are caused by an infectious agent called the prion (Prusiner 1982). In a classical infectious disease the causative agent is assembled from proteins, lipids and nucleic acids, no matter whether it is a parasite, a bacterium, a fungus or a virus. Yet, the infectious agent of TSEs, is only a protein. The prion, or PrP^{Sc} ($PrP^{Scrapie}$), is an abnormally folded, proteinase K (PK) resistant, β-sheet rich form of the endogenous cellular prion protein, PrP^{C} (Bolton et al. 1982; Prusiner 1991). That PrP^{Sc} is a misfolded version of the endogenous PrP^{C}, was allegorized in mice deficient in PrP^{C} – after challenge with else contagious prions, these mice remained healthy and did not suffer from neurodegenerative disorders (Büeler et al. 1993). FDCs not only express high levels of PrP^{C}, but also promote the accumulation of PrP^{Sc} after infection (Kitamoto et al. 1991; McBride et al. 1992; Jeffrey et al. 2000; Thielen et al. 2001; Heggebo et al. 2002)(Fig. 6). A central role of FDCs in the neuroinvasion after peripheral exposure to prions was shown in mouse models lacking mature FDCs, where disease was either abrogated completely, or delayed (Mabbott et al. 2000; Mabbott et al. 2000; Montrasio et al. 2000; Mabbott et al. 2002; Prinz et al. 2002; Mabbott et al. 2003).

Many publications suggest that after accumulation and replication of prions in the peripheral lymphoid organs, the innervations of LNs and spleen by peripheral nerves represent the entry site of prions to the CNS (Fig. 7). While sympathectomy delayed the progression of prion diseases upon peripheral administration in mice, splenic hyperinnervation resulted in an acceleration of incubation time (Glatzel et al. 2001). Furthermore, mice with FDCs juxtaposed to the splenic nerves (*Cxcr5*$^{-/-}$) show an increased velocity of prion neuroinvasion into the spinal cord (Prinz et al. 2003). The capture of prions by FDCs was shown to be dependent on the presence of complement and complement receptors. After peripheral prion infection mice lacking is CD21/35, or different complement components such as C1q, C3, or C4 had less prions accumulating in SLOs and were partially or fully protected against prion diseases (Klein et al. 2001; Mabbott et al. 2001).

DEVELOPMENT OF FOLLICULAR DENDRITIC CELLS

The induction of secondary lymphoid organs

While TLOs are generated postnatally in the presence of antigen, the SLO anlagen form during embryonic development of vertebrates. This process requires the coordinated interplay of cell populations, migratory hematopoietic cells and local non-hematopoietic stromal cells, and molecules including chemokines, adhesion molecules, and cytokines.

Members of the tumor necrosis factor (TNF) family are major molecular switches needed for LN development. Especially LT signaling was shown to be a main driver of SLO development. The lymphotoxin complex ($LT\alpha\beta_2$), consisting of one alpha and two beta subunits, interacts with the lymphotoxin β receptor (LTβR). Mice lacking LT signaling, due to genetic ablation or treatment with a soluble decoy LTβR, do not develop LNs, or PPs, and have a disturbed splenic microarchitecture (De Togni et al. 1994; Browning et al. 1995; Rennert et al. 1996; Futterer et al. 1998)

Events at the cellular level are the following. At the future sites of LNs, lymph sacs bud off from venous vessels, and blood cells start to accumulate within. It was long believed that lymph sacs are essential for the initiation of lymphocyte clustering (Sabin 1902). Recently, it was shown that in the absence of lymphatic endothelial cells (LEC) and thus lymph sacs, LN anlagen are induced, however the progression of LN development is slowed down (Vondenhoff et al. 2009). Stromal organizer cells

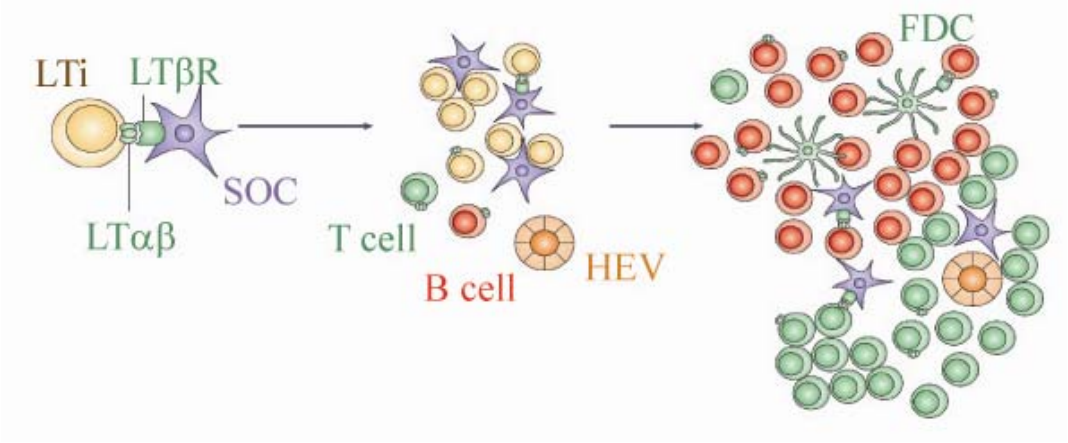


Fig. 8 Lymph node development
LTis arrive at future LN sites and interact with the local SOC, through the binding of LTαβ to stromal LTβR. Subsequent upregulation of chemokines, adhesion molecules, and lymphangiogenic factors, results in the formation of HEVs, which allow the further influx of LTis and of first B and T cells supplying the developing node with essential LTαβ. Later during development, distinct B and T cell zones are established. Adapted from Nature Reviews Immunology (Aloisi and Pujol-Borrell 2006)

(SOCs) are present at the future sites of LNs. They express the chemokine CXCL13, are positive for the adhesion molecules VCAM-1 and ICAM-1 and LTβR (Honda et al. 2001). CXCL13 expression is usually triggered by activation of the LT pathway. Nevertheless, in the very early LN development, CXCL13 is independent from LT, and seems to be regulated by other mechanisms, such as nerve activation (van de Pavert et al. 2009).

The first hematopoietic cells to arrive at the sites of future LNs, are the lymphoid tissue inducer cells (LTis). These cells are CD4 positive, but in contrast to T helper cells they do not express CD3, but $LT\alpha\beta_2$, IL-7R, RORc(γt) (Retinoid-related orphan receptor gamma) (Cupedo et al. 2002). That LTis indeed are important for the initial induction of future LNs, was shown in mice deficient in RORc(γt): here, dependent on the report LTis are absent and LNs do not form (Kurebayashi et al. 2000; Sun et al. 2000; Eberl et al. 2004). LTis cluster at sites where SOCs are present (Fig. 8), attracted by CXCL13 and adhesion molecules (Aloisi and Pujol-Borrell 2006; Vondenhoff et al. 2007). LTis then signal to the SOC via the expression of surface $LT\alpha\beta_2$ inducing a positive feedback loop. The SOCs further upregulate adhesion molecules, chemokines, and lymphangiogenic factors (Vondenhoff et al. 2009). This results in the development of HEVs and the influx of naïve lymphocytes. Furthermore, separate T and B cell zones establish as a result of specific stromal

compartments, and FDCs undergo final maturation in the presence of B cell membrane bound LT$\alpha\beta_2$.

The differentiation of follicular dendritic cells

FDCs have major functions in establishing the microarchitecture of SLOs and TLOs during steady state as well as during active adaptive immune response. They maintain tissue structure via secretion of chemokines and production of adhesion molecules. During the GC reaction they ensure affinity maturation of B cells displaying native antigen on their surface and supplying high affinity antibody bearing B cells with survival factors, while controlling the appropriate removal of dying cells and cell debris by TBMs secreting factors such as Mfge8 (Cyster et al. 2000). Still, little is known about the origin of this cell type. Major investigations were undertaken to determine signaling mechanisms in FDC maturation and maintenance. The study of knock out mice revealed that FDCs allure follicular B cells via the secretion of CXCL13, and B cells in turn support the differentiation of FDCs. Mice lacking CXCL13 or its receptor CXCR5 have mislocalized FDCs and B cell zones, and more dramatic, mice devoid of B cells do not sustain presence of FDCs (Cerny et al. 1988; Forster et al. 1996; Ansel et al. 2000). Interestingly the majority of B cells express the CXCL13 receptor CXCR5, however, other molecular cues must exist, which specify the restricted migration of certain B cell subpopulations into the follicle (Forster et al. 1994; Forster et al. 1996)). The molecular signaling events needed for the generation of FDCs could be attributed to

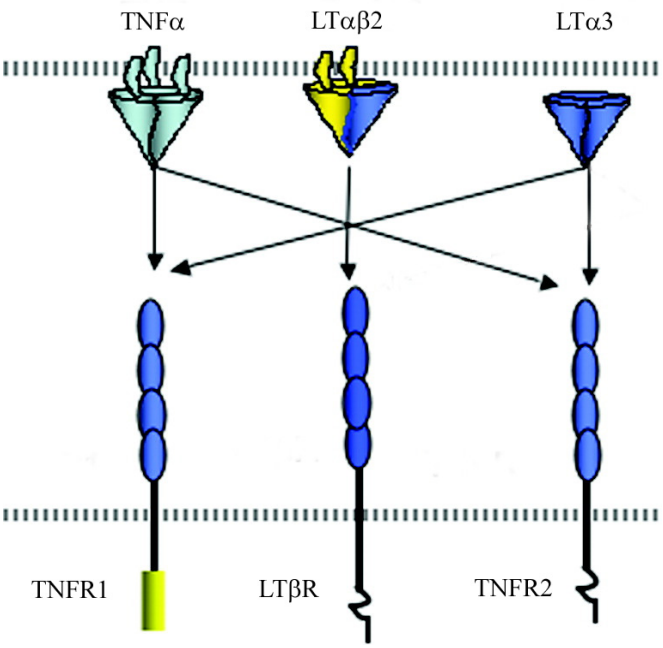


Fig. 8 Ligands and receptors of the TNF family
TNFα, LT$\alpha\beta_2$ and LTα_3 are expressed by the B cell. TNFα, LT$\alpha\beta_2$ are membrane bound, LTα_3 is secreted. LT$\alpha\beta_2$ signals via the LTβR and activated the NFκb pathway. TNFα and LTα_3 signal via TNFR1 and TNFR2. TNFR1 engagement results in activation of the NFκb as well as the death pathway. Adapted from Annual Review of Immunology (Ware 2005)

LT and TNF highly expressed by B cells (Fig. 9) (Chaplin and Fu 1998; Ware 2005). While the $LT\alpha\beta_2$ signals through LTβR, TNFα and the soluble LT alpha homotrimer ($LT\alpha_3$) interact with TNFR1 and TNFR2. Engagement of LTβR induces both NFκb (nuclear factor kappa B) pathways, the classical one, signaling via IKKαβγ complex leading to the nuclear translocation of the transcription factor RelA, as well as the

Gene Deletion	FDCs	LN	PP	Splenic Architecture
LTα	-	-	-	Disrupted
LTβ	-	+/- (MLN)	-	Disrupted
LTβR	-	-	-	Disrupted
TNFα	pre FDC in MZ?	+	-	Disrupted MZ
TNFR1	pre FDCs in MZ?	+	-	Disrupted MZ
TNFR2	+	+	+	+

Table 1 Genetic deficiencies in LT and TNF signaling pathways and correlated phenotypes
Genetic deletions are indicated in the left row. – absent, + present, FDCs: +/- FDC like cells were reported in the marginal zone of the spleen. LNs: +/- approximately 75% of MLNs present. Adapted from Annual Reviews Immunology (Fu and Chaplin 1999; Ware 2005).

non-canonical pathway via NIK-IKKα and transfer of RelB into the nucleus. TNFR1 stimulation activates the canonical NFκb pathway as well as the death signaling cascade (Ware 2005). RelA induces the transcription of inflammatory genes. RelB on the contrary is needed for the expression of genes regulating development.

Though mice devoid of TNFα or TNFR1 maintain having LNs and PPs, they lack mature FDCs and show a disrupted splenic microarchitecture (Table 1). Segregated T and B cell areas are established, but they are mislocalized, with follicular B cells accumulating underneath the marginal sinus (MS) (Le Hir et al. 1995; Le Hir et al. 1996; Pasparakis et al. 1996; Pasparakis et al. 2000). Mice with a deficiency in LTβR signaling have a more dramatic block in SLO development. In mice without LTα or LTβR, none of the LNs and PPs can be observed, whereas *Ltb*$^{-/-}$ animals retain certain LNs. The splenic white pulp is severely disorganized, T and B cells are intermingled, and no FDCs are present (De Togni et al. 1994; Koni et al. 1997; Futterer et al. 1998). The importance of LT and TNF not only in the formation of FDCs, but also in their maintenance, was allegorized in an experiment done by Mackay *et al.*, where a soluble LTβR-Ig decoy receptor was used to block endogenous signaling (Mackay and Browning 1998). This resulted in the

dedifferentiation of the mature FDCs within a matter of days. On the contrary, overexpression of LT in non-lymphoid organs such as the pancreas, kidney, skin or liver, leads to the generation of ectopic TLOs containing mature FDCs (Picarella et al. 1992; Heikenwalder et al. 2005).

As discussed some signaling switches needed for FDC development and maintenance are understood. Hardly anything however is known about the founder cell giving rise to mature FDCs. The main efforts undertaken, so far, studied whether FDCs are derived from a hematopoietic or from a non-lymphoid, mesenchymal cell. Their mesenchymal origin is supported by BM reconstitution experiments in wild type (WT) mice after lethal irradiation. These experiments show that after a reconstitution period of a year all FDCs are derived from a stromal compartment of the host animal (Humphrey et al. 1984). Grafting experiments, where splenic slices were transplanted subcutaneously into mice, showed that after 6 months the donor tissue had been populated by hematopoietic cells from the host mouse, but all FDCs remained donor derived. These results further corroborated previous results, suggesting that FDCs are derived from a local, rather sessile, and non-hematopoietic cell (Imazeki et al. 1992). Another study by the group of Szakal questions the sessile and mesenchymal origin of FDCs (Kapasi et al. 1998). Here, neonatal mice with severe combined immunodeficient (SCID), reconstituted with BM or fetal liver cells from mouse and rat donors, developed in parts FDCs that were of donor origin. BM and blood are not only a source for hematopoietic cells, but do contain mesenchymal stem cells (MSC) (Campagnoli et al. 2001; Roufosse et al. 2004). These data suggest that under certain circumstances FDCs can be derived from a migratory cell derived from the BM, be it a hematopoietic or mesenchymal one.

When postnatal development of FDCs in mouse spleens was studied using the anti-FDC-M1 antibody, cells expressing its antigen, Mfge8, were not detected before postnatal day 3 (Balogh et al. 2001; Kranich et al. 2008). These cells were appearing along peripheral parts of the emerging white pulp and were radiation resistant. The first occurrence of IC trapping cells was reported only later at the age of 2-3 weeks (Yoshida and Takaya 1991; Imazeki et al. 1994). The study of TNFR1 signaling deficient mice revealed cells with a dendritic appearance in the MZ, expressing C4 (FDC-M2) as well as CD35. These observations, however, should be considered with

the notion that these mice have a dramatic increase in MZ B cells, cells which localize to the very same area of the lymphoid follicle and stain for C4 and CD35.

Electron microscopic experiments had revealed ultrastructural differences between FDCs and other fibroblast reticular cells within the follicles (Mitchell and Abbot 1965; Heusermann et al. 1980; Yoshida and Takaya 1989; Yoshida et al. 1993). After immunization with antigen and induction of GC reactions, transitional forms between the two cell types were observed, adding evidence for a mesenchymal, and fibroblast origin of FDCs (Kamperdijk et al. 1978; Yoshida and Takaya 1989; Yoshida et al. 1993).

Even though there are many indications for a mesenchymal origin of FDCs, the true founder cell has remained as elusive as the question, whether FDCs are derived from a stromal cell, with a sessile or a migratory character.

OUTLINE OF THE STUDY

It hat so far been technically challenging to follow FDC maturation for different reasons. First of all, FDCs share many markers with other cells. e.g. CD21/35 and FcγRIIb are expressed by follicular B cells as well as MZ B cells. FDCs' main characteristic is the trapping of ICs, a function which is established by the time when FDCs are mature. IC-capturing assays can therefore not be used to follow FDC precursor differentiation. Another useful marker for FDCs is FDC-M1, however it also detects TBMs, and the antigen detected by the anti-FDC-M1 antibody had been unknown. This lack of FDC-specific markers also made a transgenic approach to follow FDC progenitors by tracing impossible.

Huber *et al.* performed a transcriptomic microarray analysis to identify FDC-specific genes. For this, the gene profile of isolated FDCs was compared to WT spleens, containing FDCs, and spleens from *Ltbr*$^{-/-}$ mice, which are devoid of FDCs. In addition RNA expression of mice depleted conditionally of FDCs by treatment with soluble LTβR-Ig fusion protein was assessed (Huber et al. 2005). One of FDC-associated marker identified by this screen was Mfge8.

Using different approaches, we could show that FDC-M1 is identical to Mfge8 (Kranich et al. 2008). Since Mfge8 is a secreted protein involved in the removal of apoptotic cells from GCs by TBMs, we hypothesized that Mfge8 positivity of TBMs could occur secondarily due to their phagocytic activity. Indeed, using different techniques including *in situ* hybridization (ISH) for Mfge8 mRNA, we could show, that all Mfge8 in SLOs was derived from stromal cells, mainly FDCs, and not macrophages. The other stromal cells producing Mfge8 were lining the marginal sinus and within the T cell area of the splenic follicle.

Further analysis revealed that these cells expressed many other FDC markers, were insensitive to irradiation, but their maintenance was LTβR-dependent. These results confirmed the close relationship of the cells to mature FDCs and were suggesting that they could be putative precursors.

Next, I assessed the presence of these possibly early stage FDCs in different mice lacking mature FDCs. I pinpointed down a role of TNFR1 and B cells in the final maturation of FDCs. The early development was mainly determined by LTβR and the common cytokine receptor gamma chain, and lymphoid tissue inducers. These results provide new insights in molecular and cellular mechanisms in FDC development.

In addition, I analyzed the expression of *Mfge8* in kidney and liver, as examples for non-lymphoid organs, where FDCs can be induced ectopically. In the kidney, Mfge8 was found in the glomerular structures, probably produced by mesangial cells. In WT livers, Mfge8 could be detected in oval cells, and expression was independent from LTβR activation. Upon treatment of mice with LTβR agonist, Mfge8 in oval cells as well as other cells of the liver was upregulated.

I furthermore studied the development of FDCs using *Mfge8* ISH in postnatal mice as well as in $Rag2^{-/-}\gamma c^{-/-}$ mice. $Rag2^{-/-}\gamma c^{-/-}$ spleens lacked follicular structures, and few $Mfge8^{+}$ cells clustered perivascularly. In these mice FDC development was induced by reconstitution with WT BM. This revealed that FDCs form in a continuous process – first, appearing and expanding at perivascular sites, later, budding off into the future B cell follicles. The same ontogenetic processes could be recapitulated studying the postnatal development. These results suggests a continuous maturation of perivascular precursors, which I termed proFDCs, into so-called preFDCs of the MS and the follicles, undergoing terminal differentiation this leads into the generation of mature FDCs. Mutant mice that had been analyzed previously, therefore, reflect the blockade of FDC maturation at certain developmental stages.

I then set out to determine the true founder cell. The perivascular appearance in the spleen suggested that FDCs might be derived from a perivascular stem cell. Pericytes are of mesenchymal origin and are reported to be stem cells for fibroblast, muscle, and fat tissue. Using a pericyte reporter mouse I determined that mature FDCs are derived from Pdgfrb-expressing cells, which probably reflect pericytes.

Furthermore, I developed tools to answer following FDC-related questions:
- what is the contribution of early Mfge8 expressing cells in SLOs to the mature FDC pool?
- which cells are the FDC precursors in non-lymphoid organs?
- is there a direct involvement of FDCs in prion replication, autoimmunity and chronic inflammations, cancer?

Therefore transgenic mice were generated to irreversibly label precursors of FDCs and facilitate the isolation of FDCs at different developmental step, as well as to gene target and conditionally ablate FDC populations. DNA constructs were made using bacterial artificial chromosome (BAC) technology, placing 5 different constructs under the control of the Mfge8 promoter. So far 4 constructs were injected into oocytes to generate transgenic mice.

RESULTS

THE DISCOVERY OF CELLS WITH RELATIONS TO FDCs

MZ Mfge8^{+} cells express markers of FDCs

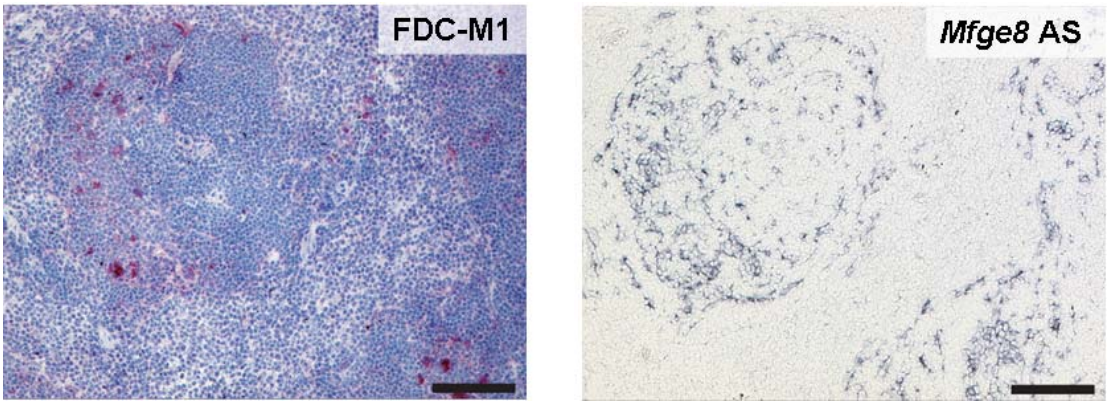


Fig. 1 Staining for FDC-M1 and RNA *ISH* for Mfge8
Consecutive sections of a WT mouse spleen where either stained for FDC-M1 (Mfge8) and counterstained with haematoxylin or hybridized with a DIG-labelled AS probe for Mfge8 and stained with anti-DIG antibodies. Mature FDC networks as well as cell in the marginal sinus and the T cell zone express Mfge8 RNA. Scale bar 100μm.

Originally, I had performed in situ hybridization (ISH) for *Mfge8* on WT splenic sections, to determine the cellular source of this secreted protein (Kranich et al. 2008). Comparing the protein staining by immunohistochemistry (IHC) with the pattern of *Mfge8* RNA detected by ISH, I realized that RNA detection not only is more specific

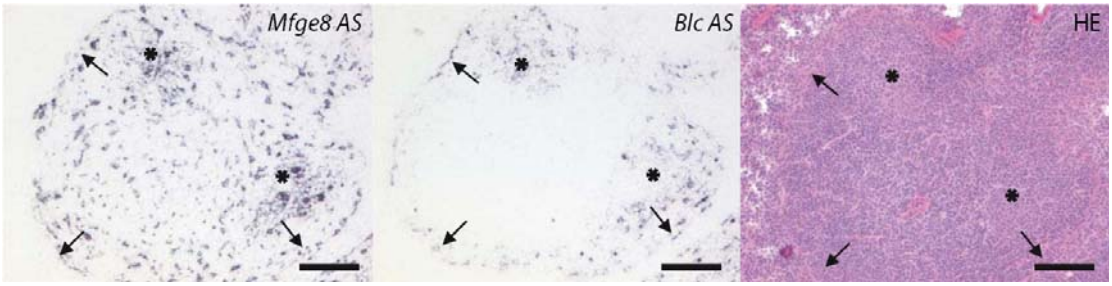


Fig. 2 RNA *ISH* for *Mfge8* and *Blc*
Consecutive sections of a WT mouse spleen where hybridized with a DIG-labelled AS probe for Mfge8 (left), respectively *Blc* (middle) or stained with HE. Mature FDC networks (*) and cells lining the MS (arrows) are positive for both markers. Scale bar 100μm.

than IHC for the protein, as TBM are not detected, but also far more sensitive. Cells in the area of MZ and within the T cell area would be readily determined by ISH (Fig.1). This suggested that these cells might be related to mature FDCs and even represent precursors (preFDCs) that had been suggested to exist in the MZ. To test this idea, I performed ISH for *Blc* RNA, a factor secreted by FDCs, and compared its expression pattern to the one of *Mfge8* (Fig. 2). The staining intensity for *Blc* on mature FDCs as well as on preFDCs was weaker, nevertheless the pattern was very much alike the ISH for Mfge8. Furthermore, co-stainings of Mfge8 with other FDC markers, including BP3 and PrP, the adhesion molecules ICAM-1 and MAdCAM, the receptors essential for the IC capturing by FDCs CD21/35 and FcγRIIb were performed (Fig.3, data not shown). ICAM-1 and MAdCAM were co-localizing well with mature FDCs as well as preFDCs (Fig. 3 A, B). Immunofluorescence for CD21/35 (Fig. 3 C), and FcγRIIb (data not shown) determined that mainly mature FDCs express these markers, but also low expression by other cells, presumably follicular and MZ B cells, was detected. BP-3 showed co-localization with FDCs and

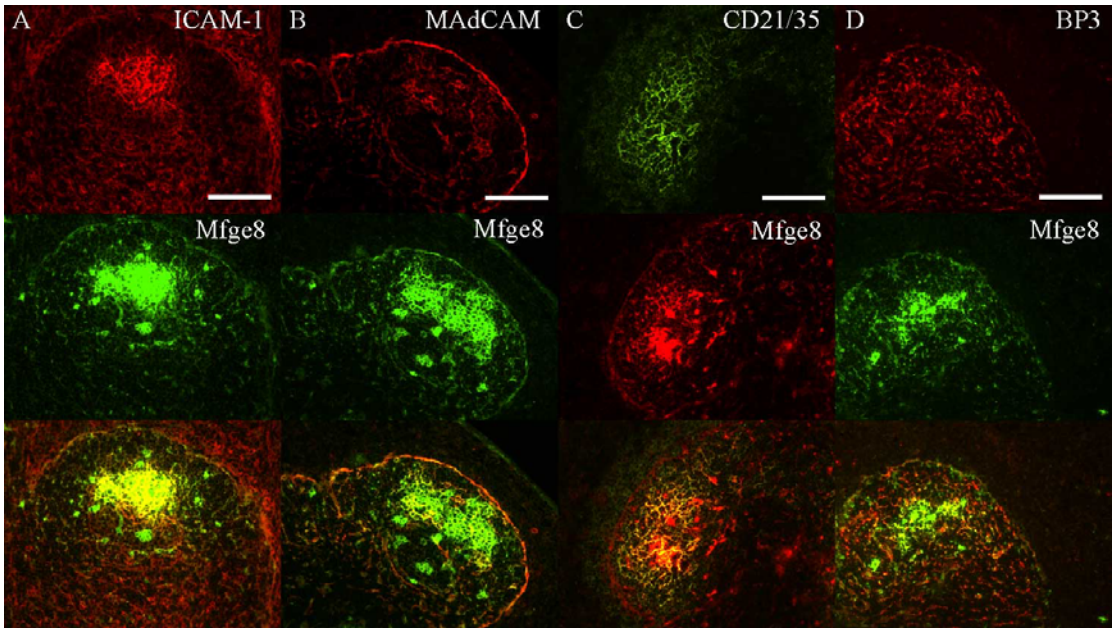
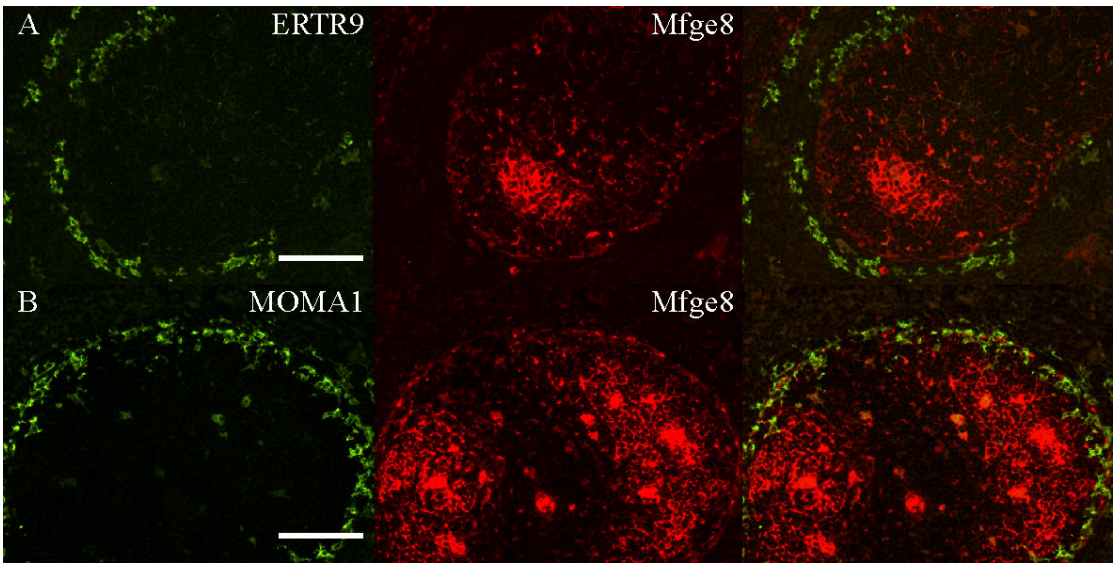


Fig. 3 FDC markers present on MS preFDCs
Splenic cyosections double stained with Mfge8 and ICAM-1 (A), MAdCAM (B), CD21/35 (C), or BP3 (D). MS preFDCs. show high expression of the FDC markers ICAM-1, and MAdCAM. Some overlay with BP3 and little immunofluorescence for CD21/35, Upper and middle row show single stainings, lower row showsoverlay. Scale bar 100um

preFDCs, but also stained additional cells in the T cell zone (Fig. 3 D). PrP was highly expressed on mature FDCs, but immunofluorescence did not detect PrP present



on other cell types (data not shown). Under certain conditions, such as thioglycollate stimulations or *in vitro* culture (data not published, not shown), macrophages were shown to upregulate Mfge8 (Hanayama et al. 2002). I therefore wanted to exclude that Mfge8 in the MZ is derived from MZ residing macrophage populations. MOMA-1^+ metallophilic macrophages and ERTR-9^+ MZ macrophages did not co-localize with Mfge8 (Fig. 4).

preFDC are radioresistant and stromal

To further substantiate the close kinship between the two cell populations, I explored the origin, and the radiation sensitivity. Therefore, I generated BM chimaeras of WT and *Mfge8*$^{-/-}$ mice. 8 weeks after engraftment, mice were analyzed for the presence of *Mfge8*$^{+}$ cells by ISH. As published already for mature FDCs (Kranich et al. 2008), all *Mfge8*$^{+}$ cells, including the MS lining cells and the cells within the T cell zone are derived from a non-hematopoietic, radiorestistant compartment (Fig. 5).

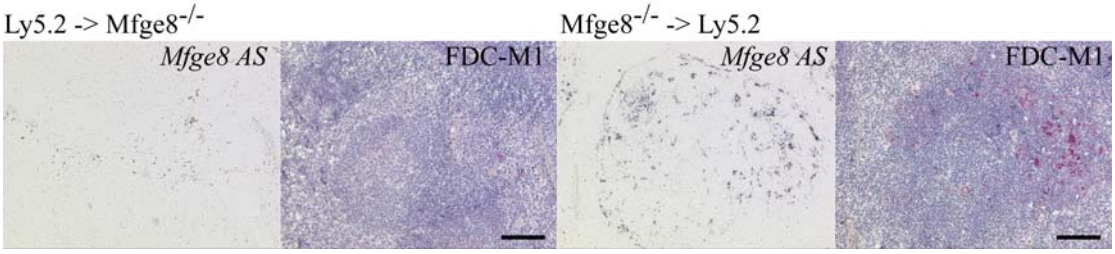


Fig. 5 *Mfge8* is made by stromal, radioresistant cells
WT and Mfge8$^{-/-}$ mice were lethally irradiated and reconstituted with BM from the Mfge8$^{-/-}$ or WT donors, respectively. Mice were immunized and boosted with Ova-Alum. Splenic sections of mice were then analyzed for the presence of Mfge8 RNA and protein using Mfge8 AS probe and the FDC-M1 antibody. Scale bar 100um.

preFDCs ARE PRESENT BEFORE MATURE FDCs EMERGE

Development of preFDCs relies on LTβR signalling but is independent from TNFR1 activation

LN and splenic development is strongly impaired by an absence of LTβR signalling: LNs do not form, and splenic architecture is heavily disturbed. The effect of TNFR1 deficiency is less pronounced. Here, LNs do develop, and the cellular composition as well as their localization less disturbed. Having this in my mind, I was wondering whether preFDC development was dependent on either receptor signalling, and analyzed spleens from *Tnfa*$^{-/-}$ (data not shown), *Tnfr1*$^{-/-}$, *Lta*$^{-/-}$, *Ltb*$^{-/-}$, and *Ltbr*$^{-/-}$ (Fig. 6). The pool of preFDCs seemed unchanged in mice lacking TNFR1 signalling. Here,

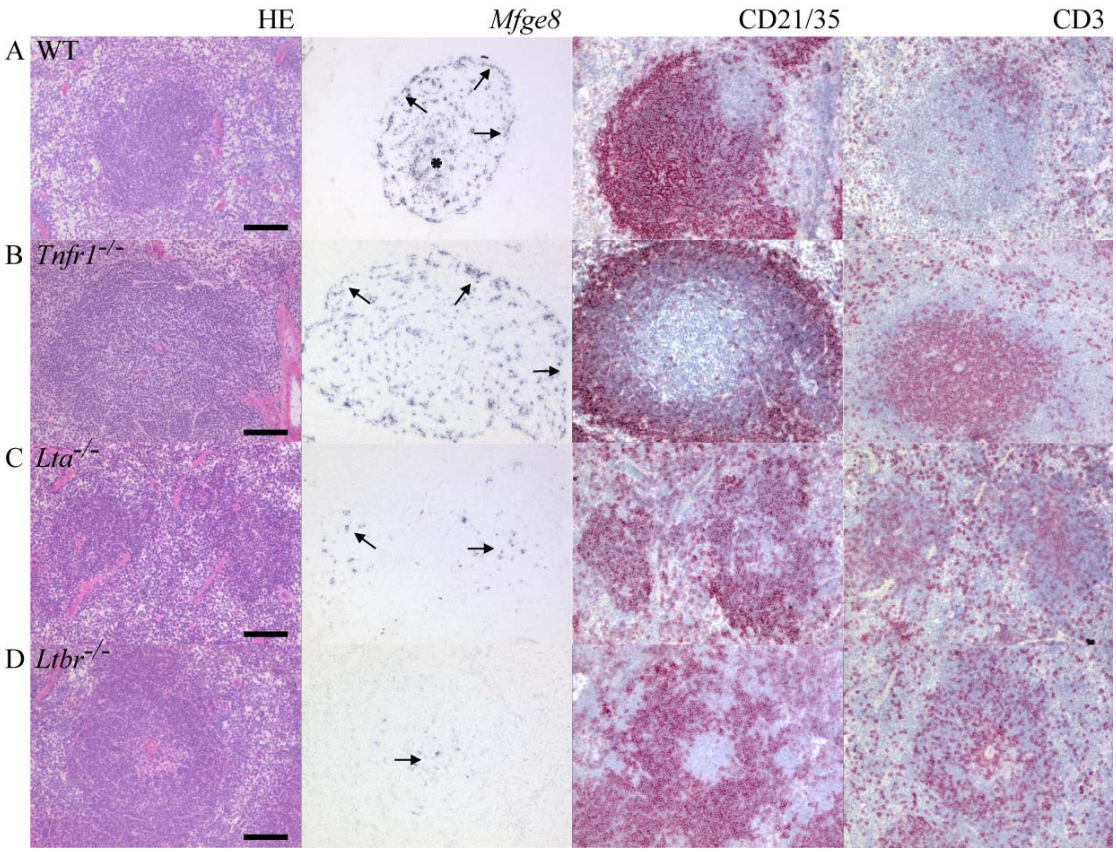


Fig. 6 Development of preFDCs depends strongly on LTβR
Consecutive sections of a WT (A), *Tnfr1*$^{-/-}$ (B), *Lta*$^{-/-}$ (C), *Ltbr*$^{-/-}$ (D) spleen where stained with HE (left), hybridized with a DIG-labelled AS probe for Mfge8 (second from left), or immunostained for CD21/35 (B cells and FDCs) and CD3 (T cells). Mature FDC networks (*) and cells lining the MS and within the follicle are indicated with arrows. Scale bar 100μm.

Mfge8^{+} cells were present in the MZ and the T cell area just like in WT spleens. The amount of cells present in mice with a LTβR signalling deficiency was drastically reduced, and the remaining *Mfge8*$^{+}$ cells were localized to white pulp follicles. A similar distribution of *Blc*$^{+}$ cells was observed in the different LT/TNF deficient mice. While *Tnfr1*$^{-/-}$ mice retained many cells lining the follicular sinus, mice with a lack in LTβR signalling, had only very few cells distributed within follicular structures (Fig. 7).

LN development rests upon presence of LT. The analysis of FDC-like cells in LNs using *Mfge8 AS* probe was therefore restricted to WT and *Tnfr1*$^{-/-}$ mice, which

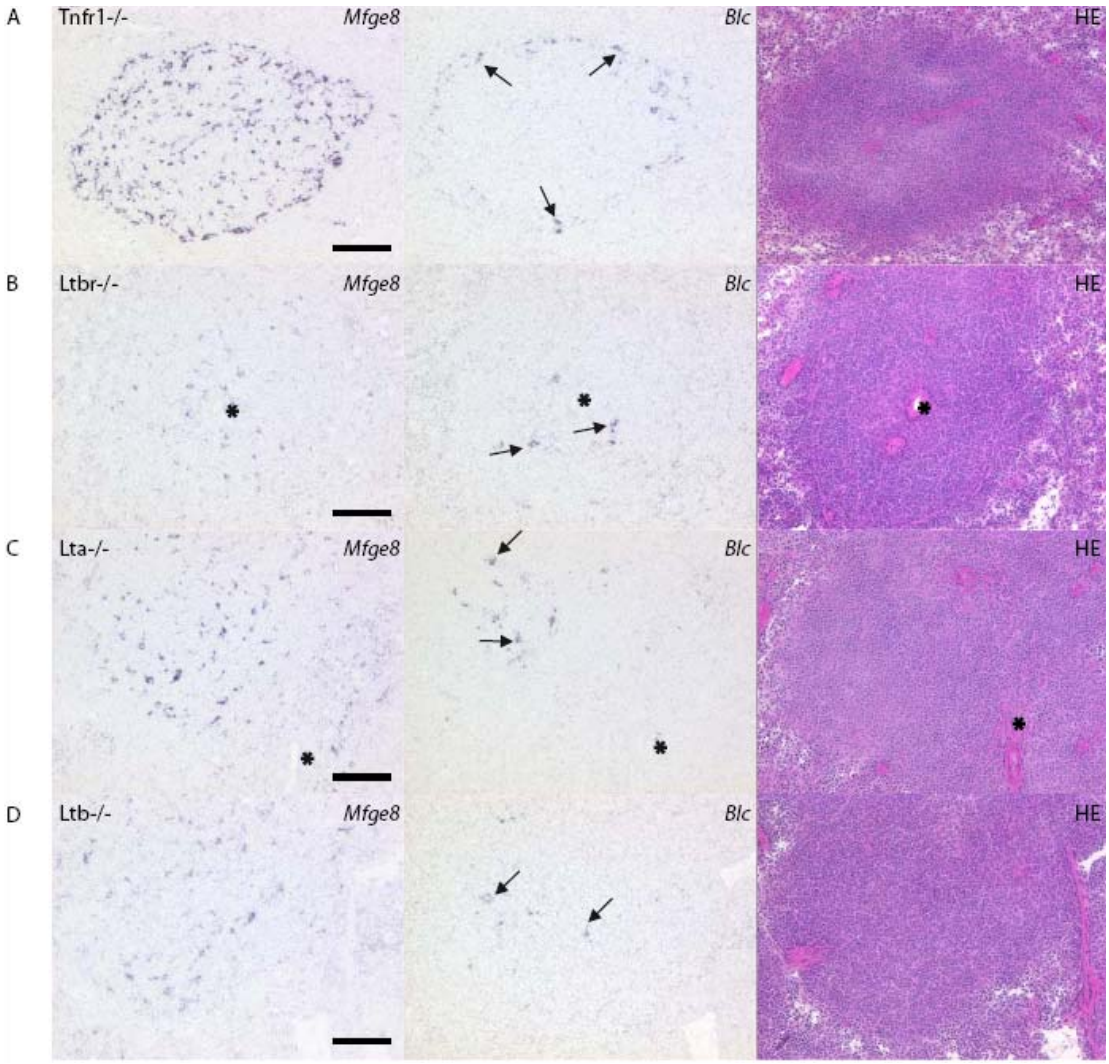


Fig. 7 Cells expressing *Mfge8* and *Blc* in TNFR1 and LTβR signalling deficient mice
ISH for *Mfge8* (left row) and *Blc* (middle row) was performed on consecutive sections of splenic sections from *Tnfr1*$^{-/-}$, *Lta*$^{-/-}$, Ltb$^{-/-}$, and *Ltbr*$^{-/-}$ mice. * indicate central arteriole. Arrows point to *Blc* expressing cells within white pulp follicles. Scalce bar 100μm.

retain LNs, however, devoid of mature FDCs. ISH revealed that not only the B cell

follicles contained *Mfge8*$^{+}$ FDC networks, but that cells positive for this RNA could be found in cortex and medulla of WT LNs (Fig. 8). Furthermore, *Mfge8*$^{+}$ cells were highly abundant in *Tnfr1*$^{-/-}$ mesenteric LNs (MLNs), indicating again that preFDCs do not depend on TNFR1 signalling. Quantitative measurements for *Mfge8* in LNs of *Tnfr1*$^{-/-}$ compared to WT mice revealed a trend in reduction of *Mfge8* in MLNs from *Tnfr1*$^{-/-}$ of approximately 30%, though this was not significant (Fig. 8). Only ILNs had a significant reduction to 80% of WT ILN. I concluded from these results, that though TNFR1 activation is a main factor needed for the generation of mature FDCs, preFDCs in spleens and LNs develop independently. Especially MLNs maintained a very high expression of *Mfge8*

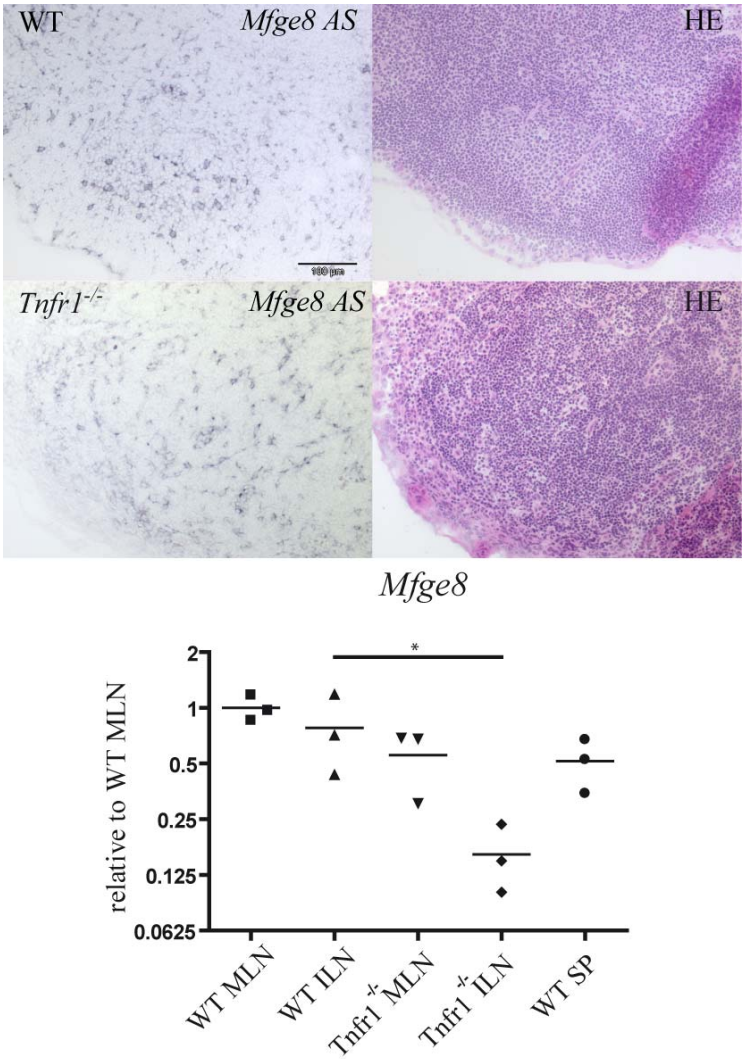


Fig. 8 FDCs and preFDCs in LNs of WT and *Tnfr1*$^{-/-}$ mice
ISH for *Mfge8* on WT (upper left) and *Tnfr1*$^{-/-}$ (lower left) MLN. Consecutive sections were stained with HE. ISH not only detected cells in the B cell follicles but also lining the subcapsular sinus and all over the cortex and medulla of the LN. Scale bar 100µm. Graph shows relative amounts of *Mfge8* mRNA from WT and *Tnfr1*$^{-/-}$ inguinal LN and MLN normalized over *Gapdh* and *Mfge8* levels of WT MLN. In total the relative expression in LNs is slightly higher than in a WT spleen (SP). Unpaired-t test * $p \leq 0.05$.

levels in the absence of TNFR1. In contrast to peripheral LNs, MLNs were shown to even develop in the absence of LTβ underlining their uncommon development. In line with this is our observation that the expression of the FDC marker *Mfge8* in Tnfr1$^{-/-}$ remained largely unaffected. The relative amount of *Mfge8* in WT LNs was increased compared to splenic tissue. This is most probably due to the fact that splenic tissue is

made of lymphoid, but also non-lymphoid compartments. Though total amount of white pulp content of a spleen is higher than in a LN, the relative expression of white pulp markers is reduced.

preFDCs' maintenance in WT and *Tnfr1*$^{-/-}$ depends on LTβR activation

LTβR signalling can be blocked using a soluble decoy LTβR fused to an immunoglobulin domain (LTβR-Ig) competing with the endogenous receptor for the ligands. Treatment of mice for 24h with LTβR-Ig leads to dedifferentiation of FDCs, including the loss of classical FDCs markers and trapped antigen in the spleen (Mackay and Browning 1998). Furthermore, marginal sinus MAdCAM^{+} cells are absent after blocking of LTβR. In mice treated with a decoy TNFR1, the amount of bound ICs is diminished as well, nevertheless, mature FDC networks remain (Mackay and Browning 1998). I was therefore interested to see whether the maintenance of

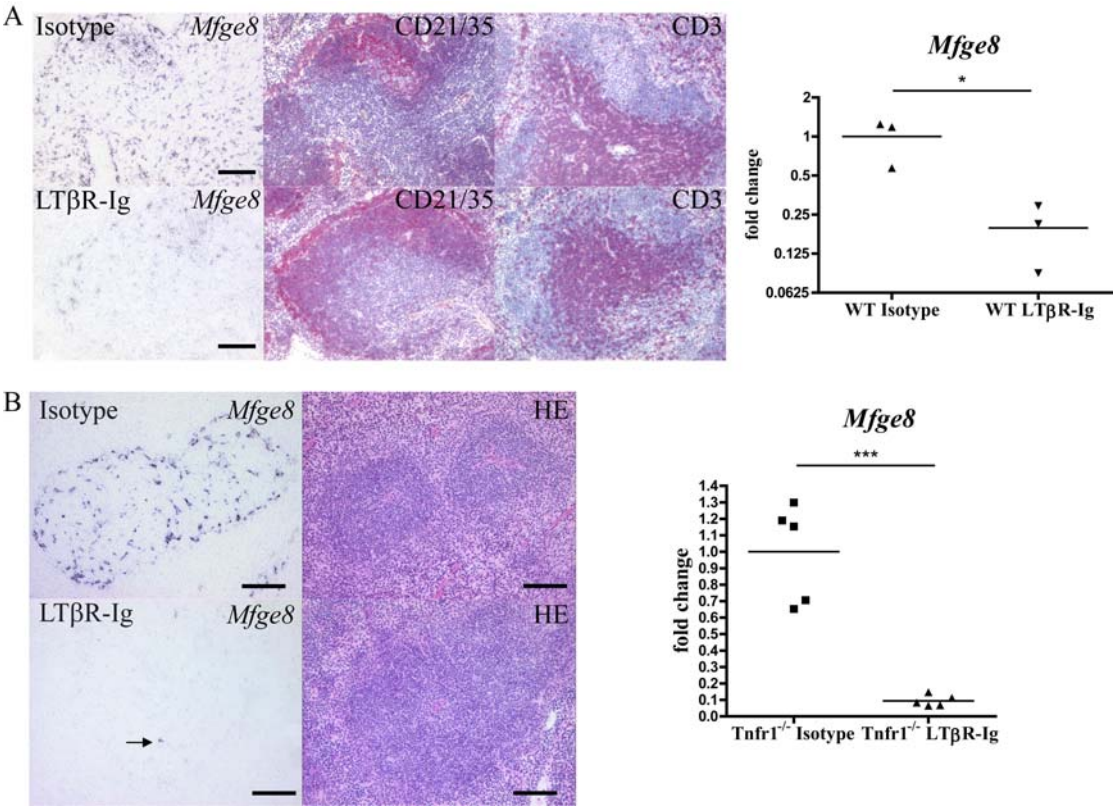


Fig. 9 Maintenance of preFDCs depends on the expression of LTβR
(A) WT mice were treated for three weeks with 100µg LTβR-Ig or respective isotype control i.p, weekly. ISH for *Mfge8* was carried out on splenic cryosections, consecutive sections were stained by IHC for CD21/35 and CD3. *Mfge8* RNA is down-regulated to 20% (0.2±0.1; n=3) compared to *Mfge8* expression in isotype treated WT mice (fold change 1±0.37). (B) Treatment of *Tnfr1*$^{-/-}$ mice for 1 week with 100µg LTβR-Ig or respective isotype i.v (n=5). Spleens were analyzed by *Mfge8* ISH and qPCR. Blocking LTβR activity lead to a 90% reduction in Mfge8 expression (fold change 0.09±0.03) compared to isotype treated (fold change 1.00±0.30). Unpaired-t test * p≤0.05, *** p≤0.001. Scale bar 100µm.

preFDCs depended on the persistent activation of LTβR signalling and treated WT as well as Tnfr1$^{-/-}$ mice with soluble LTβR -Igs or isotype control. Blockage of LTβR

activation resulted in the loss of preFDCs in, both, WT and Tnfr1$^{-/-}$ mice (Fig. 9). FDCs are important for the proper localization of follicular B cells, attracting them via BLC. Interestingly, it had been previously shown that the adhesion molecules ICAM-1 and VCAM-1 are important for the localization of MZ B cells, and furthermore that LTβR signalling was needed for the ICAM-1 and VCAM-1 expression in the spleen and treatment with LTβR-Ig would hence result in the dislodgement of MZ B cells. Since preFDCs are expressing ICAM-1 (Fig. 3) they could be the cells responsible for

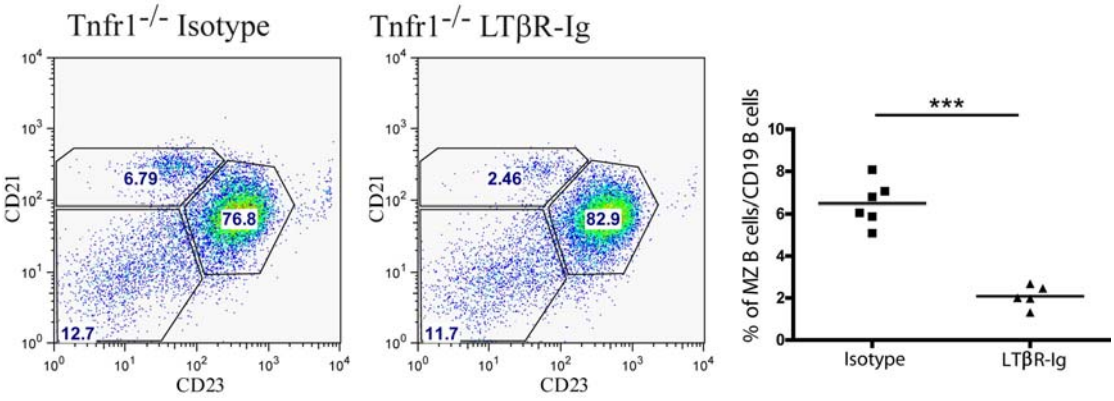


Fig. 10 MZ B cells depend on LTβR activation
Tnfr1$^{-/-}$ mice were either treated with 100µg LTβR-Ig (n=5) or hamster isotype (n=6) i.v. for 1 week. Percentage of MZ B cells (CD19^{+}CD21^{hig}hCD23^{low}) of total B cells (CD19) was determined. *Tnfr1*$^{-/-}$ LTβR-Ig treated mice (mean % 2.1±0.5) had significantly reduced amounts of MZ B cells compared to isotype treated mice (mean % of MZ B cells 6.5±1). Unpaired t-test *** p≤0.001

the accumulation of MZ B cells. Blocking of LTβR signalling in a WT spleen, as it was done by the group of Cyster, however, not only alters the cellular composition in the MS, but also leads to the dedifferentiation of FDCs. I therefore wanted to test, whether the dedifferentiation of preFDCs, only, would also result in the release MZ B cells. For this purpose I treated Tnfr1$^{-/-}$ mice either with LTβR-Ig or isotype control and determined the percentage of MZ B cells (CD21high/CD23low) per total B cells (CD19) by FACS (Fig. 10). Indeed the loss of preFDCs in Tnfr1$^{-/-}$ mice correlated with a loss of MZ B cells by approximately 60%, supporting the hypothesis that preFDCs are regulating the lodgement of MZ B cells.

preFDCs are present in mice lacking lymphocytes

The abolishment of parts of the TNF superfamily affected the development of FDC-like cells to different extents. Lymphocytes, especially B cells, are the major cell type supplying the SLOs with $LT\alpha\beta_2$, $LT\alpha_3$, and TNFα. I had observed that LTβR activation was needed to maintain preFDCs as well as MZ B cells in WT and *Tnfr1*$^{-/-}$ mice. On the other hand, the presence of B cells, supplying SLOs with LTs, is needed for FDCs to develop and to be maintained as was shown in mice lacking B cells by conditional targeting or in mice where B cells were ablated temporarily using anti-IgM antibodies (Cerny et al. 1988). Hence, MZ B cells might be involved in the establishment and maintenance of preFDCs. Compared to follicular B cells, MZ B cells express increased amounts of integrin receptors, α4β1 (VLA-4) and αLβ2 (LFA-1). *In vitro* adhesion tests had shown that these integrins on the MZ B cells mediate the attachment to the adhesion molecules VCAM-1 and ICAM-1. *In vivo* the injection of antibodies interfering with the binding to just one of the adhesion molecules, is insufficient to release MZ B cells. However, injection of antibodies blocking both simultaneously, results in the loss of this cell population within few hours, and re-emergence of MZ B cells is blocked for more than 4 days (Lu and Cyster 2002). To test whether the presence of MZ B cells was needed for preFDCs' maintenance, I ablated MZ B cells using either a combination of anti-integrin αL/VCAM-1 or anti-integrin αL/α4 antibodies (Fig. 11). After 4 days, mice were sacrificed, and the spleens assessed for the presence of MZ B cells by determining the ratio of $CD21^{high}CD23^{low}CD19^{+}$ MZ B cells over total $CD19^{+}$ B cells by FACS and the amount of *Mfge8* RNA as well as the distribution of *Mfge8*$^{+}$ preFDCs by qPCR and ISH. After blocking ICAM-1 and VCAM-1 interactions the amount of MZ B cells was drastically reduced, surprisingly, total levels of *Mfge8* or the distribution of FDCs and preFDCs remained unaltered. This result could indicate that though MZ B cells are important for preFDC, their ablation using the antibodies was incomplete or too short term to result in a dedifferentiation of preFDCs or on the other hand could suggest that preFDCs do not depend on MZ B cells after all.

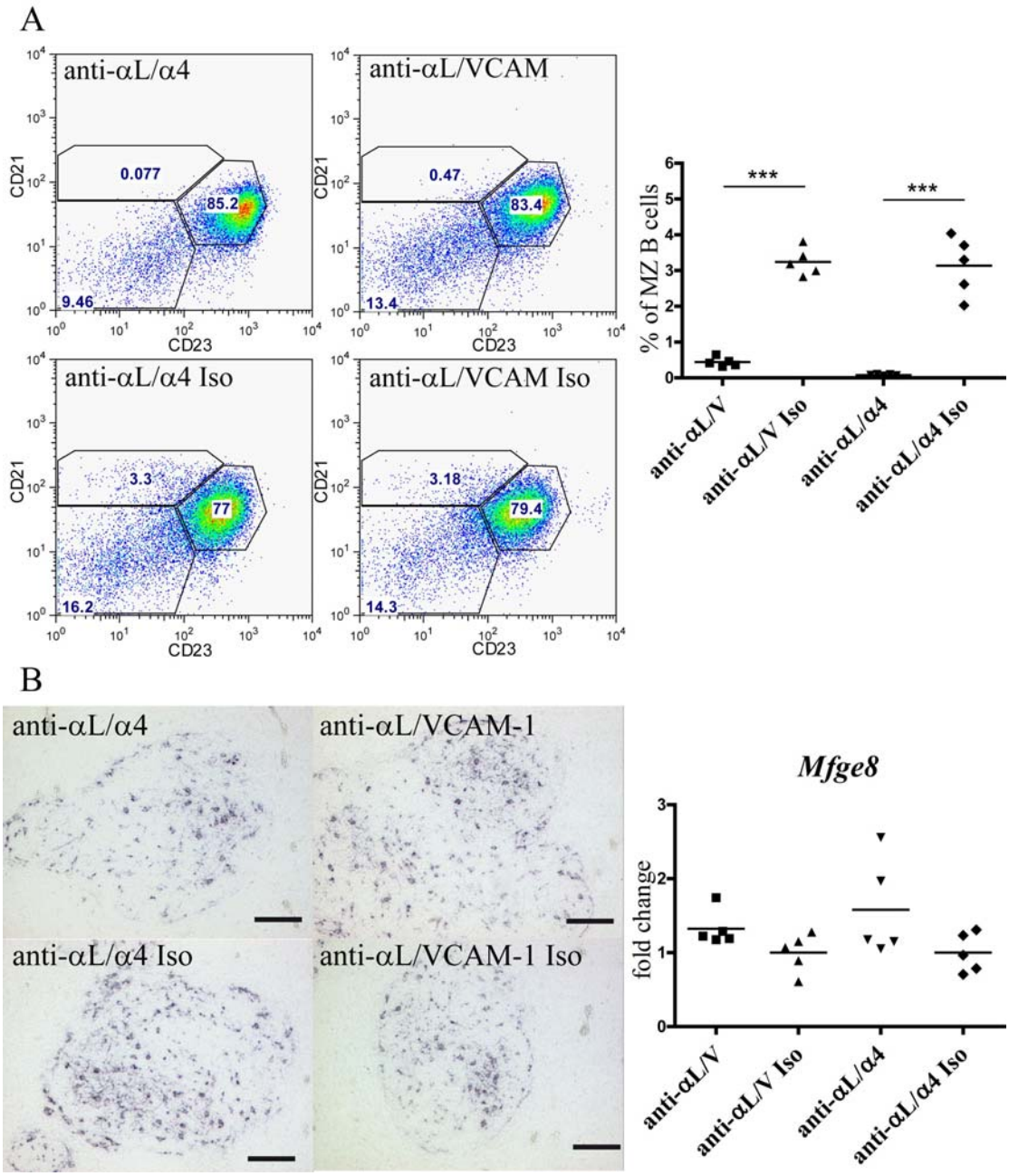


Fig. 11 Ablation of MZ B cells does not affect preFDCs

Mice were treated for 4 days with 100µg of anti-integrin αL in combination with anti- integrin α4 (anti-αL/α4) or anti-VCAM-1 (anti-αL/VCAM or anti-αL/V) antibodies or the respective isotype (iso) controls (n=5 each group). Mice were sacrificed and spleens analysed by FACS (A) or histology and qPCR (B). Percentage of MZ B cells ($CD19^{+}CD21^{hig}hCD23^{low}$) of total B cells (CD19) was determined. *Tnfr1*$^{-/-}$. anti-αL/VCAM-1 treated mice (mean % of MZ B cells 0.4±0.1) and anti-αL/α4 treated (mean % of MZ B cells 0.1±0.0) had significantly reduced numbers compared to their respective isotype treated mice (mean % of MZ B cells in anti-αL/VCAM-1 iso 3.2±0.2, in anti-αL/α4 iso 3.2±0.8). (B) ISH for *Mfge8* on splenic cryosections of WT mice treated with different antibody combinations. Right panel qPCR for relative expression of Mfge8 in spleens from VCAM/ICAM blocked mice normalized over isotype treated mice. Differences were not significant. Unpaired t-test *** p≤0.001

Because lack in TLR signalling was reported to affect the microarchitecture of the splenic MZ, with decreased amounts of MZ B cells, I investigated spleens of mice lacking MyD88, an adaptor protein needed for the downstream signalling events of TLR (Fig.12) (Prinz et al. 2003). The distribution of preFDCs in these mice however remained unaltered, thus long term changes in the MZ microarchitecture do not affect preFDCs either.

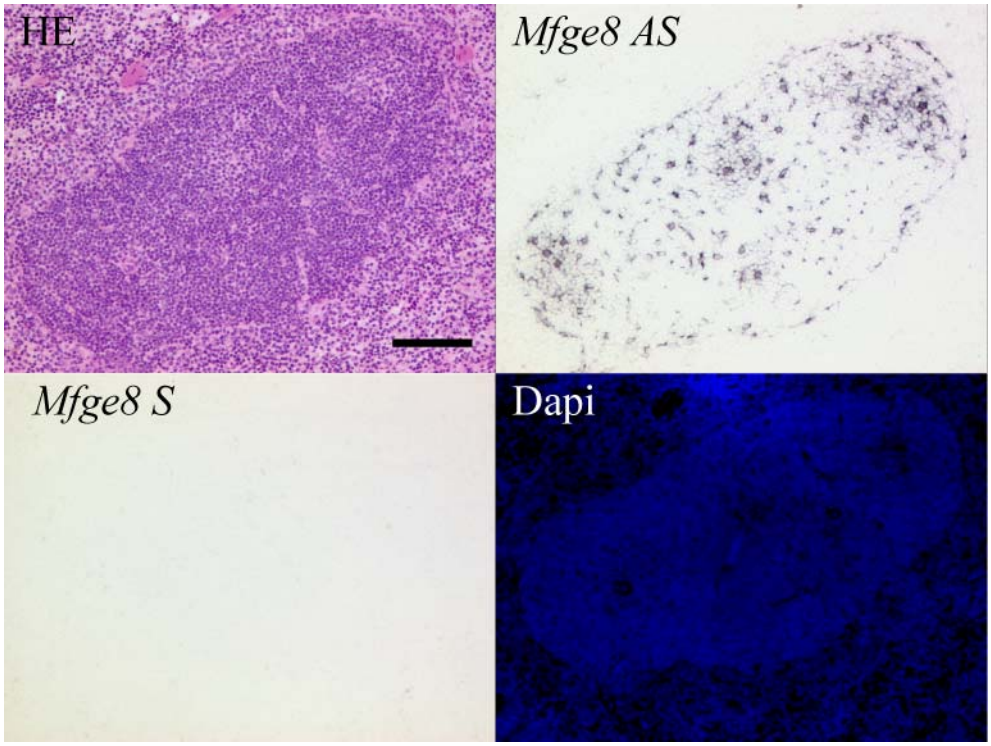


Fig. 12 Presence of preFDCs in Myd88$^{-/-}$ spleens
Spleens from Myd88-deficient mice were analyzed by ISH for *Mfge8* for the presence of preFDCs. Consecutive sections were stained with HE or with the Mfge8 sense probe as a negative control (*Mfge8 S*), Dapi staining war performed on the section hybridized with *Mfge8 S* probe.

The ablation of MZ B cells thus had no effect on preFDCs. MZ B cell however only constitute the minor amount of splenic B cells, and the remaining B cells might supply preFDCs with factors including LTαβ. We therefore investigated the role of a constitutive ablation of all B lymphocytes, analyzing μMT^{D} mice (deletion of the membrane domain of IgM heavy chain results in a developmental block), but also the consequence of the ablation of both, B and T cells, ($Rag1^{-/-}$: lacking the recombinase activating gene 1) on the development of putative FDC precursors. ISH for *Mfge8* and *Blc* was performed on splenic sections from these knock out mice (Fig. 13 and Fig.

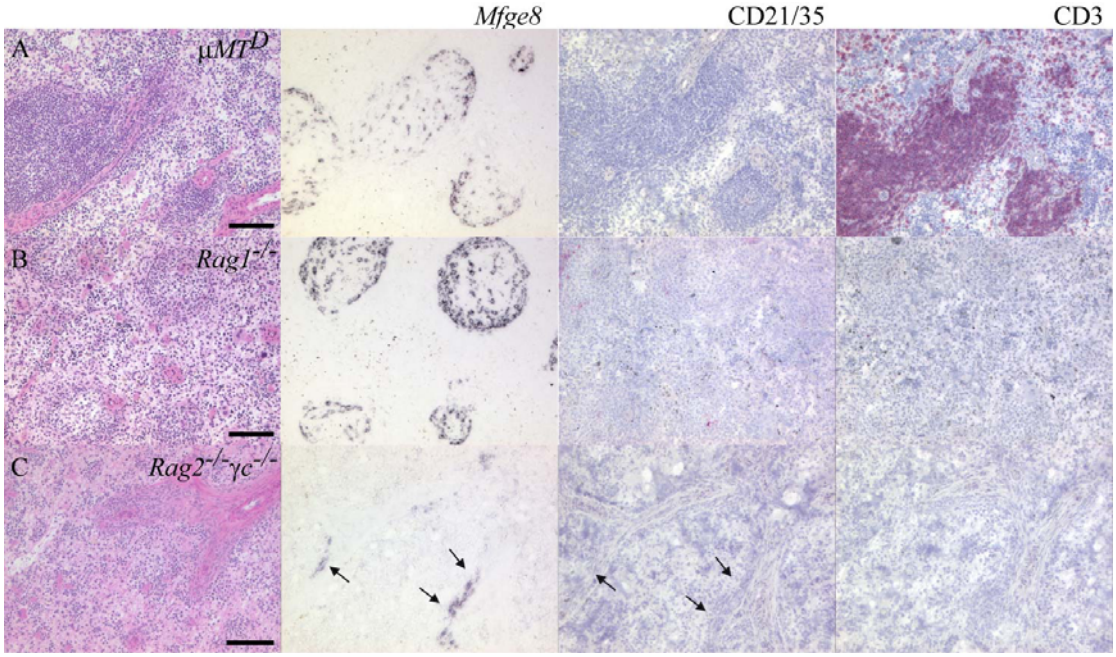


Fig. 13 *Mfge8* ISH on splenic sections of mice lacking lymphocyte populations
Mice lacking B cells (μMT^{D}, A), B and T cells ($Rag1^{-/-}$, B) B, T and NK cells ($Rag2^{-/-}\gamma c^{-/-}$, C) were analyzed for the presence of *Mfge8*+ preFDCs and proFDCs (second row left) by performing *Mfge8* ISH on splenic sections. Consecutive sections were stained with HE, CD21/35 (B cells) and CD3 (T cells). Arrows indicate proFDC clusters. Scale bar 100μm.

14). Despite the diminished size of white pulp follicles in μMT^{D} and $Rag1^{-/-}$ spleens, preFDCs were retained, thus other cells than B and T cells must be relevant for the development of preFDCs. To test whether ablation of further cell populations would affect the presence of preFDCs, $Rag2^{-/-}\gamma c^{-/-}$ spleens were investigated. In addition to Rag2 (Recombinase activating gene 2), these mice lack the common cytokine receptor

gamma chain (γc). γc is part of the interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15 receptor, and its absence leads in a developmental block of B and T cells as well as loss of all NK cells (Cao et al. 1995; DiSanto et al. 1995; Ohbo et al. 1996). *Rag2*$^{-/-}$*γc*$^{-/-}$, furthermore, do not develop any LNs and have no follicular structures in the spleen. The spleens of these mice had strongly reduced numbers of *Mfge8*$^{+}$*Blc*$^{+}$ cells, residual

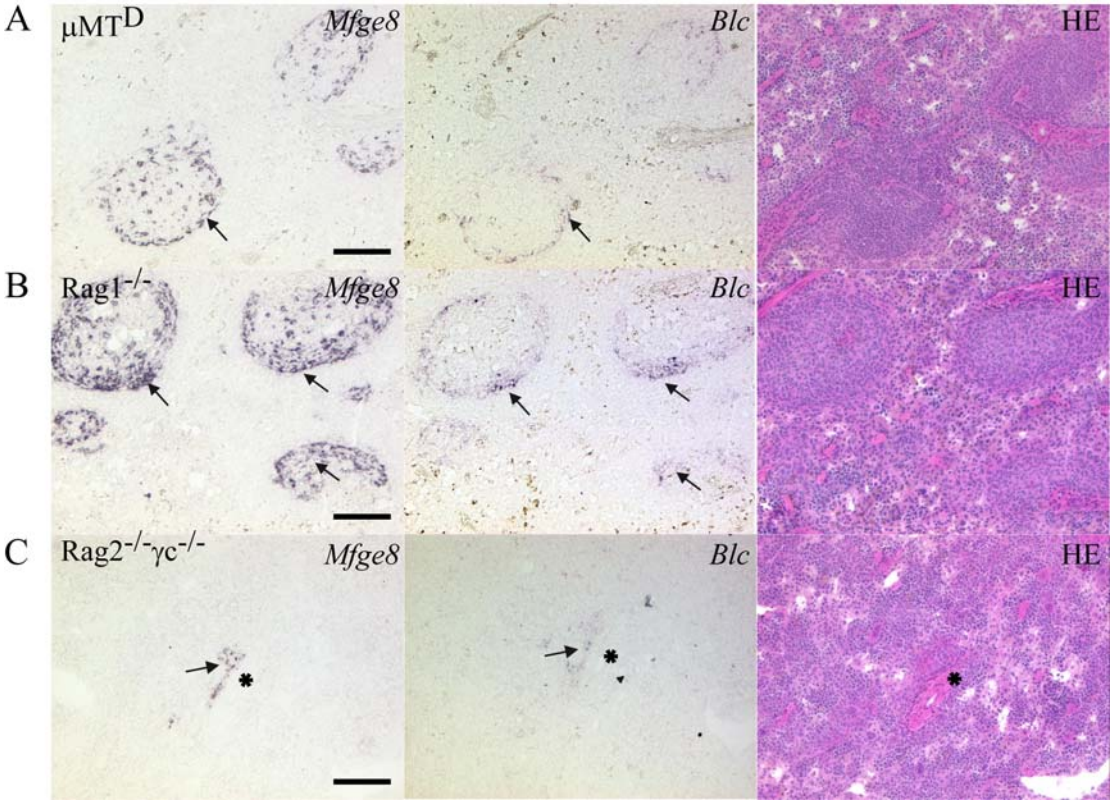


Fig. 14 proFDCs and preFDCs in mice lacking lymphocytes express *Mfge8* and *Blc*
Consecutive sections from μMT^{D}, Rag1$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ were hybridized with antisense probes against *Mfge8* and *Blc* or stained with HE. * indicate vessels, arrows point to *Mfge8*$^{+}$ and *Blc*$^{+}$ proFDCs. Scale bar 100μm.

ones were clustering around vascular structures (Fig. 9 C, Fig. 10 C). To distinguish between the MS and follicular preFDCs, and the less developed perivascular *Mfge8*$^{+}$*Blc*$^{+}$ cells of *Rag2*$^{-/-}$*γc*$^{-/-}$ mice, we termed the latter proFDCs. These results suggest, that in contrast to mature FDCs, preFDC development is not only independent from TNFR1 signalling, but even occurs in mice lacking B cells, thus in the absence of the cell, providing the major amount of local TNFα, LTα, and LTαβ. Even though, T cells were also reported to produce some LTαβ, the additional ablation of T lymphocytes did not seem to alter pre FDC distribution.

Precursor FDCs are blocked at two different stages

ISH had already shown that putative FDC precursors are present to different degrees in the different mouse mutants. I therefore wanted to assess, whether quantitative measurements could be used to determine distinct development stages. Thus I isolated RNA from splenic tissue and generated complementary DNA (cDNA) to quantify by

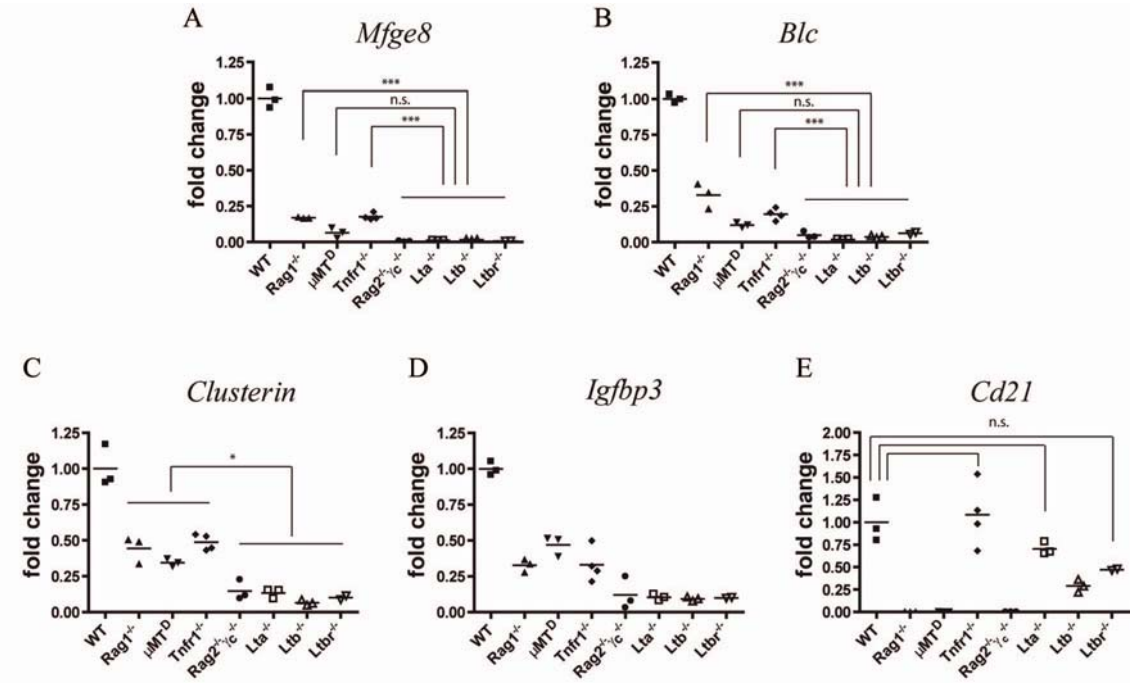


Fig. 15 Expression of FDC markers determined by quantitative RT PCR
Spleens from WT and different mutants were isolated, purified RNA was reverse-transcribed (RT) into cDNA. qPCR for *Mfge8* (A), *Blc* (B), *Clusterin* (C), *Igfbp3* (D), *Cd21* (E) were performed n=3 except *Ltbr*$^{-/-}$ n=2. Graphs show relative expression to WT spleen, all PCR results were normalized over Gapdh. unpaired t-test. A-D all mutants show significant reduction compared to WT (not indicated). n.s. not significant * p≤0.05, ** p≤0.01, *** p≤0.001

quantitative PCR the amount of FDC markers to be expressed in splenic tissue from the respective mutants and compared it to the WT expression pattern. FDC marker to be chosen, were Mfge8 and BLC to confirm the results obtained by ISH, as well as Clusterin and Igfbp3, FDC-specific mRNAs as published previously (Huber et al. 2005), as well as the complement receptor Cd21, needed for IC-trapping by mature FDCs. *Mfge8*, *Blc*, *Clusterin* and *Igfbp3*, showed all a significant reduction in the mutant mouse strains compared to WT mice (Fig. 15 A-D). Mutant mice segregated into two groups according to the expression of these RNAs. *Rag1*$^{-/-}$, *μMT*^{D}, and *Tnfr1*$^{-/-}$ showed higher expression of these FDC markers than *Rag2*$^{-/-}$*γc*$^{-/-}$, *Lta*$^{-/-}$, *Ltb*$^{-/-}$, and *Ltbr*$^{-/-}$, reflecting the more severe developmental block in the latter group. In contrast Cd21 expression behaved very differently (Fig. 15 E). While *Tnfr1*$^{-/-}$expressed at WT

levels, *Lta*$^{-/-}$, *Ltb*$^{-/-}$, and *Ltbr*$^{-/-}$ had a reduction to some extent (ranging from 25-75% of WT levels). In *Rag1*$^{-/-}$, μMT^{D}, and *Rag2*$^{-/-}\gamma c^{-/-}$ mice no apparent *Cd21* expression was detectable, indicating that even though *Rag1*$^{-/-}$, and μMT^{D} mice have the more developed preFDCs present, these cells do not express the FDC marker *Cd21* yet, but that *Cd21* expression is confined to B cells and mature FDCs.

LT is decreased in Rag2$^{-/-}\gamma c^{-/-}$ and LTβR activation stimulates Mfge8 expression

To understand the cellular signalling events which advance the development from pro to preFDCs as it is seen in *Rag1*$^{-/-}$ compared to *Rag2*$^{-/-}\gamma c^{-/-}$, we assessed differences in mRNA expression by qPCR. Because the main signalling pathways involved in LN development, in the generation of the splenic microarchitecture and FDCs are based on TNF family members, we analyzed the expression of *Lta* and *Tnfa* RNA, the ligands needed for LTβR and TNFR1 activation (Fig. 16 A and B). *Rag1*$^{-/-}$, and μMT^{D}

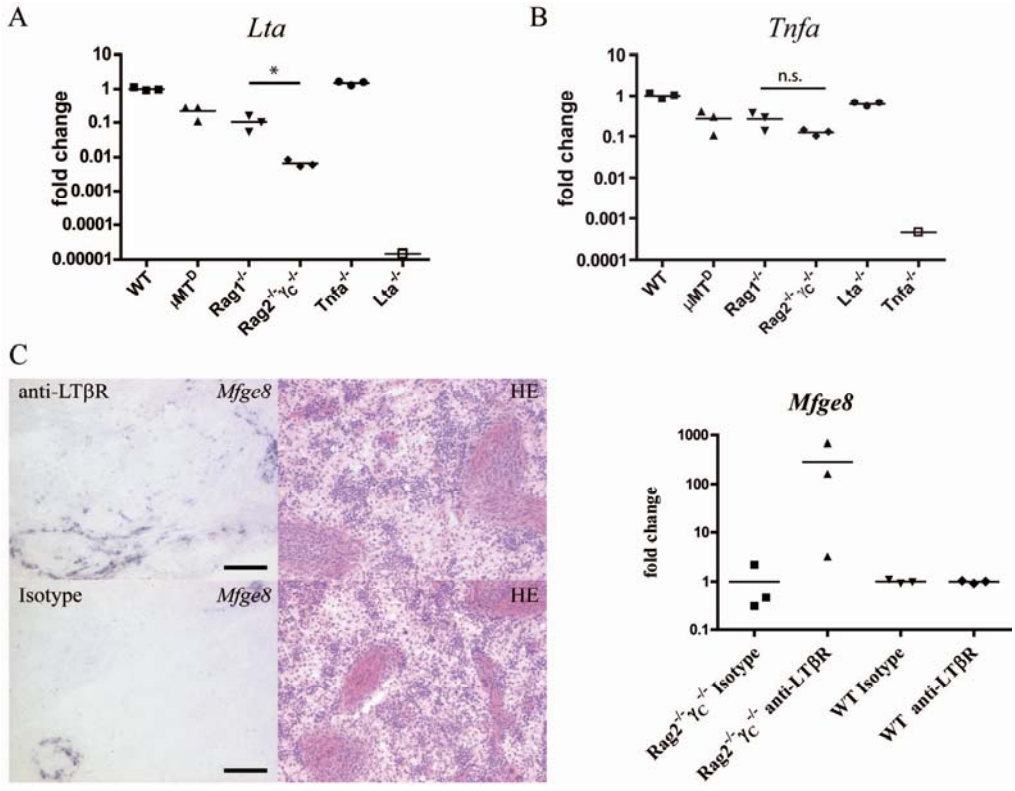


Fig. 16 LTβR activation is essential for *Mfge8* expressing cells in Rag2$^{-/-}\gamma c^{-/-}$
(A, B) Comparison of *Lta* and *Tnfa* mRNA expression in spleens from indicated mutants by qPCR. *Lta*$^{-/-}$, and *Tnfa*$^{-/-}$ spleens were used to determine threshold of detection. Unpaired t-test. n.s. not significant * $p \leq 0.05$. (C) Treatment of *Rag2*$^{-/-}\gamma c^{-/-}$ and WT mice with agonistic LTβR antibody (AC.H6) or hamster isotype control, 50μg i.v. for 24h. Spleens were analyzed for *Mfge8* expression by qPCR, normalized over *Gapdh* and isotype control. Difference was not significant according to unpaired t-test.

had an apparent reduction of *Lta* and *Tnfa* expression to 10-20% of WT levels, confirming the important role of B cells in supplying SLOs with ligands for LTβR and TNFR1. Reduction of mRNA for both TNF family ligands to 10% of WT expression thus correlated with the reduction of several FDC markers to 15-50% of WT (Fig. 15). Analysis of the *Rag2*$^{-/-}\gamma c^{-/-}$ showed that spleens from these mice had an even more dramatic loss in *Lta* mRNA to less than 1% of WT expression, but *Tnfa* mRNA remained nearly unaltered compared to *Rag1*$^{-/-}$. This indicated that LTβR activation and not TNFR1 activation is needed for the first expansion of proFDCs. To test this hypothesis, *Rag2*$^{-/-}\gamma c^{-/-}$, lacking LTβR signalling, and WT mice, sufficiently supplied with LTβR activation, were stimulated for 24h with 50ug of an agonistic anti-LTβR antibody. Next, splenic *Mfge8* expression in the spleen was determined (Fig. 16 C). Agonist treatment upregulated *Mfge8* mRNA in *Rag2*$^{-/-}\gamma c^{-/-}$ spleens approximately 250-fold compared to isotype treated mice. In WT mice, however, no change in expression was observed, indicating that the antibody cannot compete with the endogenous ligands for the binding to LTβR or signalling is at its maximum. The results obtained from the *Rag2*$^{-/-}\gamma c^{-/-}$ treated with an agonistic anti-LTβR antibody indicate that LT signalling is already essential for early pro to preFDC development.

Natural killer cells are dispensable for preFDCs

LT signalling seemed to majorly affected in *Rag2*$^{-/-}\gamma c^{-/-}$ mice, while *Rag1*$^{-/-}$ could maintain 10% of the WT expression, which was sufficient to keep preFDCs differentiated. The cellular source of LT in mice lacking B cells remained unknown.

γc-deficiency results in the absence of NK cells, and activated human NK cells were reported to express LT (Ware et al. 1992). Therefore, we wondered whether the difference between *Rag1*$^{-/-}$ preFDCs and *Rag2*$^{-/-}\gamma c^{-/-}$ proFDCs was due to a lack of NK cells. To test this, we depleted NK cells from *Rag1*$^{-/-}$ mice treating them for 3 weeks with an anti-NK1.1 antibody (Strick-Marchand et al. 2008). Depletion was confirmed by FACS using NK1.1 and DX5 as pan-NK markers, the NK population was reduced to less than 1% of the total lymphocytes, while in isotype control treated mice

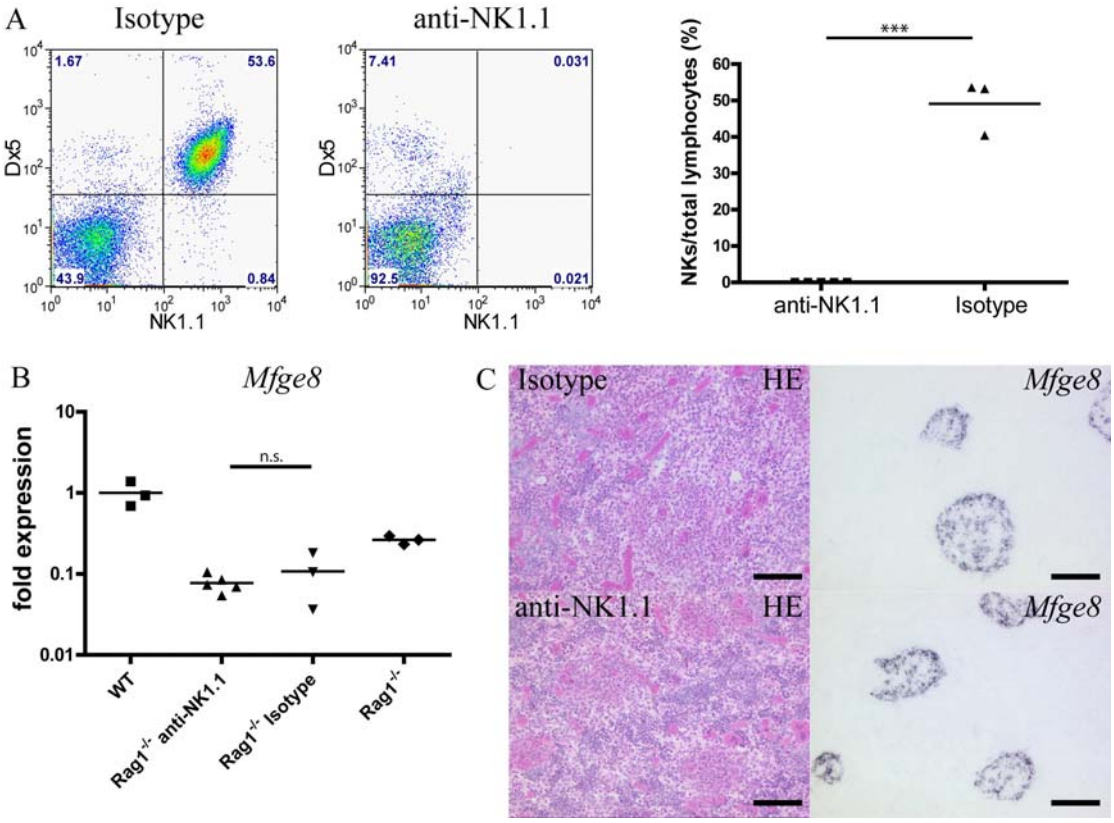


Fig. 17 preFDCs are independent from NK cells
Ablation of NKs from *Rag1*$^{-/-}$mice using anti-NK1.1 or isotype control antibody applied weekly over a time of 3 weeks. Spleens were analyzed for the depletion of NK cells by FACS using NK1.1 and pan-NK marker DX5 (A, left). Picture is representative for the respective group. Quantification of NK1.1/DX5+ cells over total lymphocyte population shows the very significant loss of NK cells (A, right). NK1.1 treated group n=5, isotype n=3. (B) Relative *Mfge8* expression in the two groups to WT expression and *Rag1*$^{-/-}$. Unpaired T-test. n.s. not significant, *** p≤0.001. ISH for Mfge8 in *Rag1*$^{-/-}$ treated with isotype antibody (upper row) or NK depleted (lower row). Consecutive sections were either HE stained or hybridized with *Mfge8* AS probe.

approximately 50% of the lymphocytes were NK cells (Fig. 17 A). Expression of *Mfge8* mRNA was determined in anti-NK1.1 antibody and isotype control treated groups (Fig. 17 B). No significant difference was detected. However, treatment with either of the antibodies resulted in a slight downregulation of *Mfge8* as determined by qPCR, suggesting that the antibody per se had an unknown regulatory effect on *Mfge8* expression. ISH for *Mfge8* showed that both groups had remaining follicles containing preFDCs, supporting that the absence of NK cells did not alter the amount of preFDCs nor their localization (Fig. 17 C).

LTis are altered in *Rag2*$^{-/-}\gamma c^{-/-}$ and LTis determine the presence of preFDCs in *Rag1*$^{-/-}$

Common cytokine deficiency leads to a signalling deficiency of a multitude of IL-Rs, including the receptors for IL2, IL-7, and IL15. More recently it could be shown that

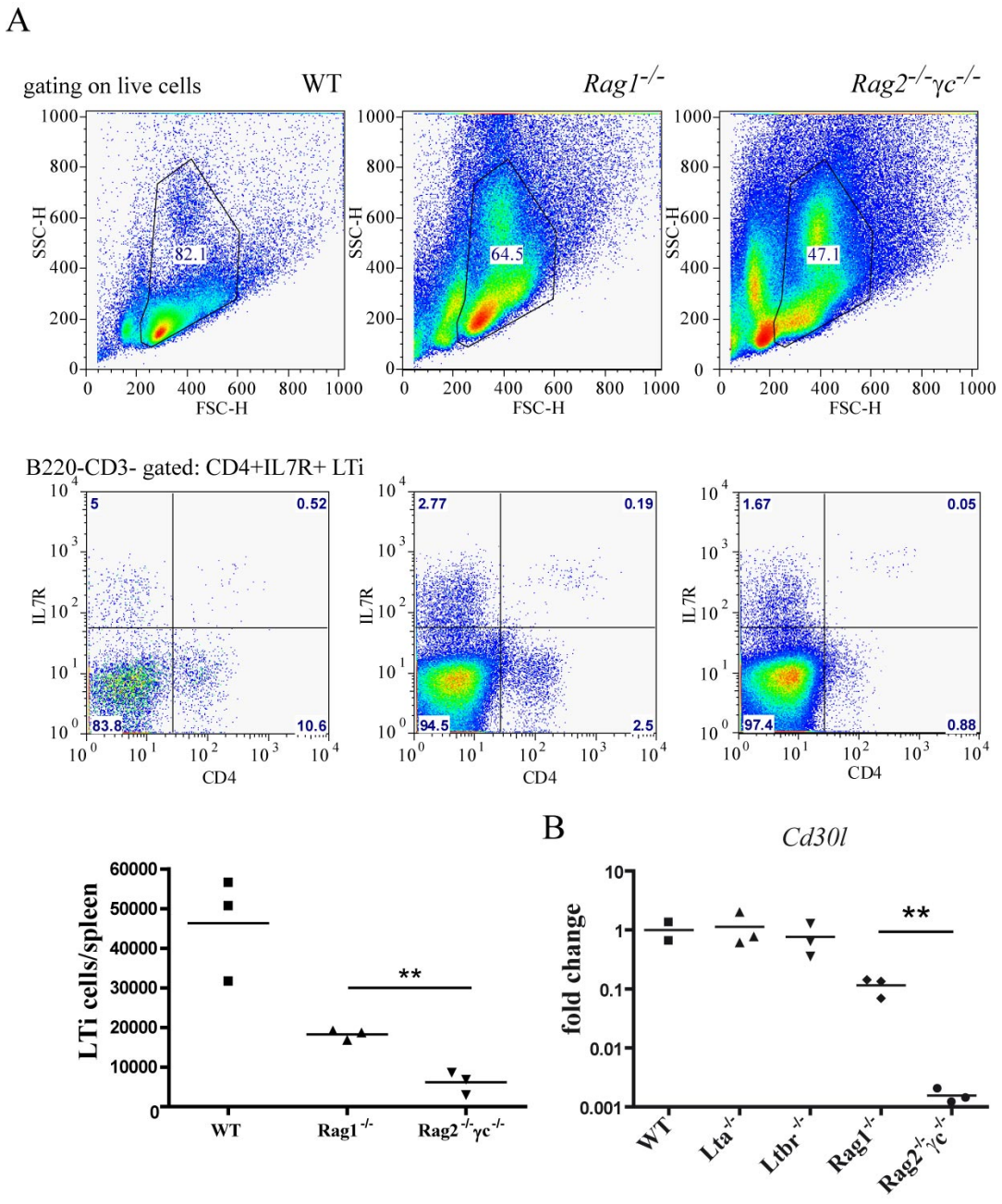


Fig. 18 Determining the number of LTis in WT, *Rag1*$^{-/-}$, *Rag2*$^{-/-}\gamma c^{-/-}$ spleens
(A) Spleens from WT, *Rag1*$^{-/-}$, and *Rag2*$^{-/-}\gamma c^{-/-}$ were analyzed by FACS for the presence of LTis. Figures show a representative picture of each group (n=3). The live gate was set using FSC/SSC (upper row), within the live gate, CD3+ and B220+ cells were excluded, and relative numbers of CD3-B220-CD4+IL-7R+ LTis are shown in dot blot (middle row). Total numbers of LTis over live cells from the spleen were then calculated (lowest row). (B) Determination of LTi specific *Cd30l* RNA by qPCR shows significant reduction in Rag2$^{-/-}\gamma c^{-/-}$ spleens. Unpaired t-test ** $p \leq 0.01$

signalling via IL2R, but more importantly IL15R is crucial for the generation of NK cells, while IL-7R is dispensable for NK cells development (He and Malek 1996; Ohteki et al. 1997). The IL-7R is highly abundant on lymphoid tissue inducer cells (LTis), the cells needed for the induction of LNs and PPs (Cong et al. 2001; Fukuyama et al. 2002; Cupedo et al. 2004; Eberl et al. 2004). Spleens of mice lacking LTis, develop normally, but contain more lymphocytes that WT spleens (Zhang et al. 2003). LTis express LTαβ, and recently it was found that deficiency of the IL-7Rα chain results in a reduction of total LTi numbers, and that cells with higher expression of IL-7R have more LTαβ present on their surface (Luther et al. 2003). In line with this, the total LTi number and the expression of cytokines such as CD30L in *Rag2*$^{-/-}$ $\gamma c^{-/-}$ was reported to be reduced when compared to *Rag1*$^{-/-}$ mice, adding additional evidence for LTis' dependence on IL-7R- signalling (Kim et al. 2005; Takatori et al. 2009). We determined total LTi numbers isolated from WT, *Rag1*$^{-/-}$, and *Rag2*$^{-/-}$$\gamma c^{-/-}$ and confirmed a reduced amount in *Rag2*$^{-/-}$$\gamma c^{-/-}$ spleens (Fig. 18). Quantification of splenic *Cd30l* mRNA revealed a significant loss in *Rag2*$^{-/-}$$\gamma c^{-/-}$ indicating that LTis are dysfunctional.

To test whether preFDCs depend on LTis, mice lacking LTis by gene targeting of the retinoid-related orphan receptor gamma (Rorc(γt)$^{GFP/GFP}$) were analyzed by *Mfge8*

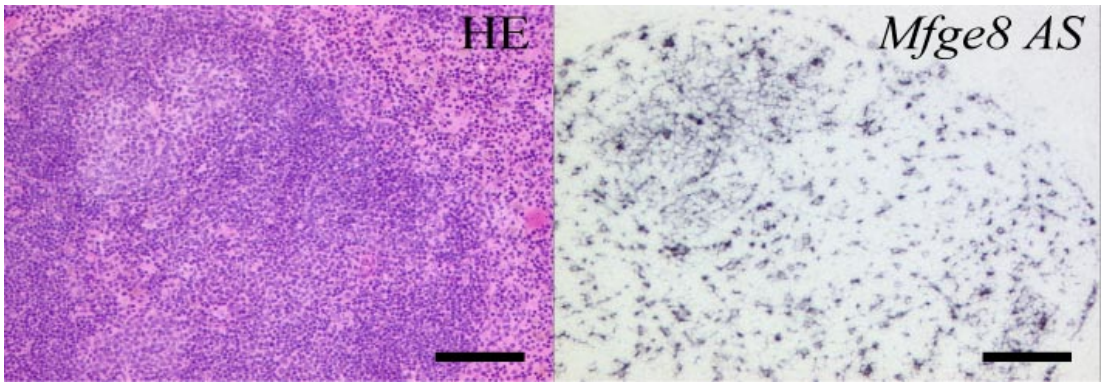


Fig. 19 FDCs and preFDCs develop normally in the absence of RORγt
Splenic sections from a Rorc(γt)$^{GFP/GFP}$ mouse was either hybridized with *Mfge8 AS* probe or stained with HE. ISH results shows that Rorc(γt)$^{GFP/GFP}$ have normal development of mature FDC networks as well as of the preFDC compartment. Scale bar 100µm.

ISH (Eberl et al. 2004). Though LTis are present in WT spleens, Rorc(γt)$^{GFP/GFP}$ mice were reported to have a normal microarchitecture of the spleen. We could also confirm that they contained mature FDC clusters as well as preFDCs around the MS as well as in the T cell zone, hence LTi function is not needed for the development of the splenic microarchitecture in WT mice (Fig. 19). In contrast to LNs, where

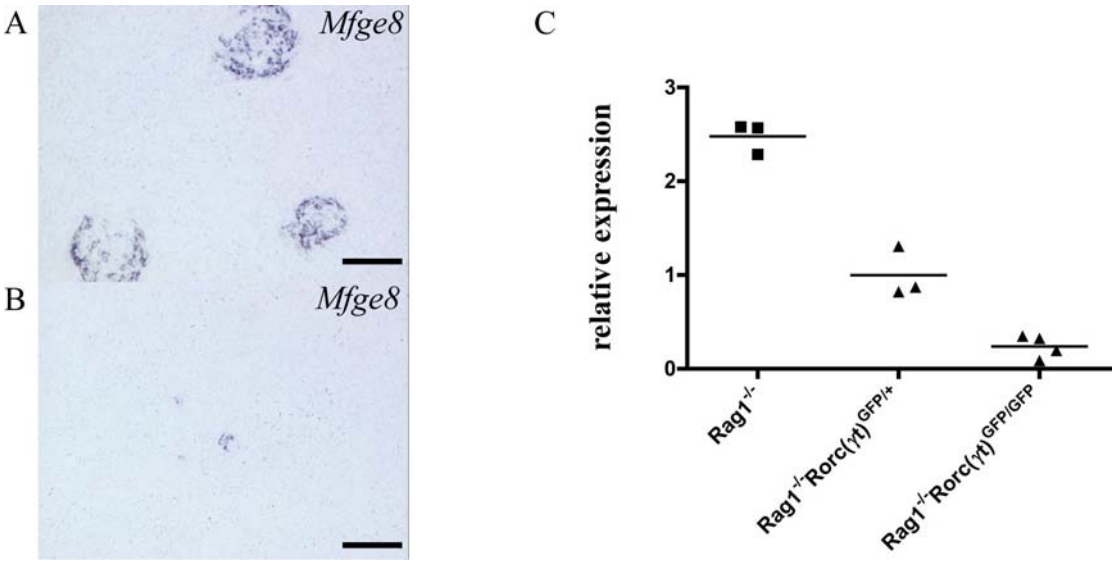


Fig. 20 preFDCs depend on LTis in the absence of lymphocytes
Splenic sections from Rag1$^{-/-}$Rorc(γt)$^{GFP/+}$ (A) were compared to Rag1$^{-/-}$Rorc(γt)$^{GFP/GFP}$ (B) were hybridized with an *Mfge8 AS*. ISH results shows that Rag1$^{-/-}$Rorc(γt)$^{GFP/GFP}$ still maintain follicle like structures, similar as in Rag1$^{-/-}$ (Fig. 13), which are not maintained in Rag1$^{-/-}$Rorc(γt)$^{GFP/GFP}$ lacking all follicular structures. Here, only few single cells expressing Mfge8 can be detected. Scale bar 100µm. (C) Relative expression of splenic Mfge8 expression in Rag1$^{-/-}$Rorc(γt)$^{GFP/GFP}$ compared to Rag1$^{-/-}$ Rorc(γt)$^{GFP/+}$ normalized over Gapdh. Unpaired t-test determined a significant reduction $p \leq 0.01$

embryonic LT made by LTis is needed to induce their development, the white pulp of the spleen develops postnatally, at a time point, when naïve B cells start to be present. The function of LTis in the spleen might therefore be compensated by B lymphocytes, expressing ligands of the TNF family. In the absence of B cells, the remaining cellular source of TNF family ligands is likely to be the LTi. To assess whether LTis are required for the presence of preFDCs present in *Rag1*$^{-/-}$ mice, *Rag1*$^{-/-}$ mice were backcrossed to Rorc(γt)$^{GFP/GFP}$ mice and compared to Rorc(γt)$^{GFP/+}$ littermates (Fig. 20). Indeed mice lacking LTis in the absence of lymphocytes showed a dramatic loss in preFDCs, and follicular structures were no longer apparent. qPCR confirmed the reduction in FDC markers. Thus, the presence of LTis results in the generation of follicular structures and the maturation of pro to preFDCs.

FDCs HAVE A PERIVASCULAR ORIGIN

Analysis of the FDC development in BM reconstituted *Rag2*$^{-/-}\gamma c^{-/-}$

Now that the cellular factors and signaling pathways were resolved we were interested in the localization of the earliest proFDC and their development and the maturation of FDCs. To follow the ontogeny of FDCs *in situ*, *Rag2*$^{-/-}\gamma c^{-/-}$ mice were reconstituted with BM derived from WT donors, to supply them with all the lymphocyte

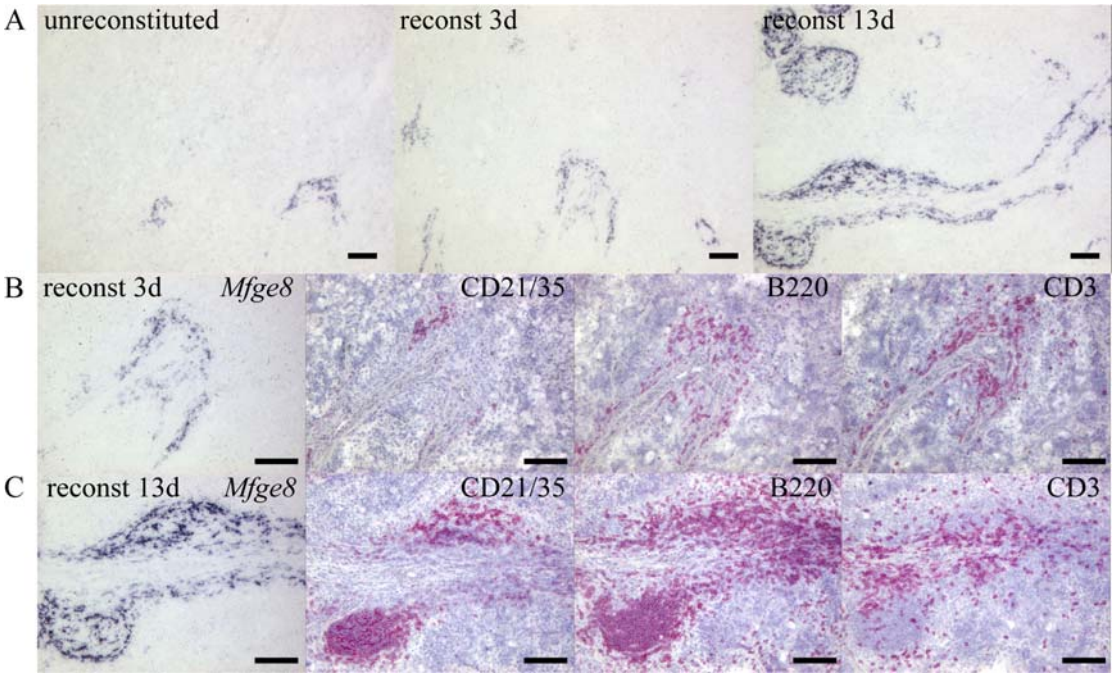


Fig. 21 Induction of FDC development in *Rag2*$^{-/-}\gamma c^{-/-}$ mice by WT BM transfer
Rag2$^{-/-}\gamma c^{-/-}$ mice were reconstituted with WT BM without irradiation and mice sacrificed at different days after transfer of BM. *Mfge8* ISH was performed on these mice to determine the FDC development status (A). Consecutive sections at day 3 post BM transfer (B) and at day 13 (C) were stained with the B and FDC cell marker CD21/35, B220, for T cells CD3 staining was performed. Scale bar 100μm.

populations and LT, which are usually absent in these mice. This environment in turn supports the development of splenic FDCs, which I set out to follow the days after BM transfer. Mice were analyzed starting from day 2 onwards by *Mfge8* ISH as well as by IHC for CD21/35, B220 and CD3 to follow the influx and maturation of lymphocytes and FDCs (Fig. 21). As mentioned previously, unreconstituted *Rag2*$^{-/-}\gamma c^{-/-}$ mice have proFDC clusters localizing next to vascular structures. Upon intravenous (i.v.) transfer of BM, lymphocytes entered the spleen at the site of proFDCs. Hand in hand with the arrival of lymphocytes I determined an increase in proFDCs at perivascular sites. proFDCs were observed along long stretches of vessels. At later

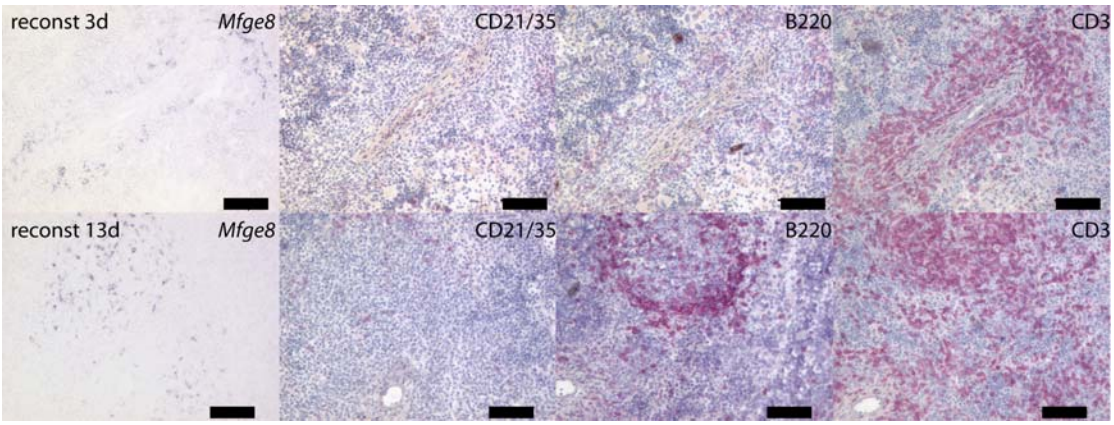


Fig. 22 Absence of preFDC development in *Rag2*$^{-/-}\gamma c^{-/-}$ mice by *Lta*$^{-/-}$ BM transfer
Rag2$^{-/-}\gamma c^{-/-}$ mice were reconstituted with *Lta*$^{-/-}$ BM without irradiation and mice sacrificed at different days post transfer of BM. Mfge8 ISH was performed on these mice to determine the FDC development status (A). Consecutive sections at day 3 post BM transfer (B) and at day 13 (C) were stained with the B and FDC cell marker CD21/35, B220, for T cells CD3 staining was performed. Though lymphocytes were infiltrating at sites where proFDCs were present, the influx of B cells was reduced compared to T cells and the upregulation of the mature B cell marker CD21/35 was strongly delayed. proFDCs did not expand efficiently. Scale bar 100μm.

time points, perivascular accumulations of FDC precursors and lymphocytes started budding off from vascular structures and generate white pulp (WP) follicles. In *Rag2*$^{-/-}\gamma c^{-/-}$ mice reconstituted with *Lta*$^{-/-}$ BM, the B cell influx was delayed, few of them expressed CD21/35. proFDCs were localized within the developing follicles, their population, however, did not expand markedly (Fig. 22). These results corroborate that the very early induction of proFDCs depends on LTβR activation, and that in its absence the positive feedback loop of chemokines and signaling molecules between hematopoietic and stromal compartment is impaired.

Postnatal development of FDCs

Because the development of FDCs upon reconstitution *Rag2*$^{-/-}\gamma c^{-/-}$ mice with WT BM might not reflect events that occur under normal circumstances, we decided to analyze the development of FDCs in the developing WP of WT mice. In contrast to the LNs, the splenic WP is not formed during embryogenesis of the mouse, but develops postnatally. We therefore analyzed the development of FDCs by *Mfge8* ISH starting

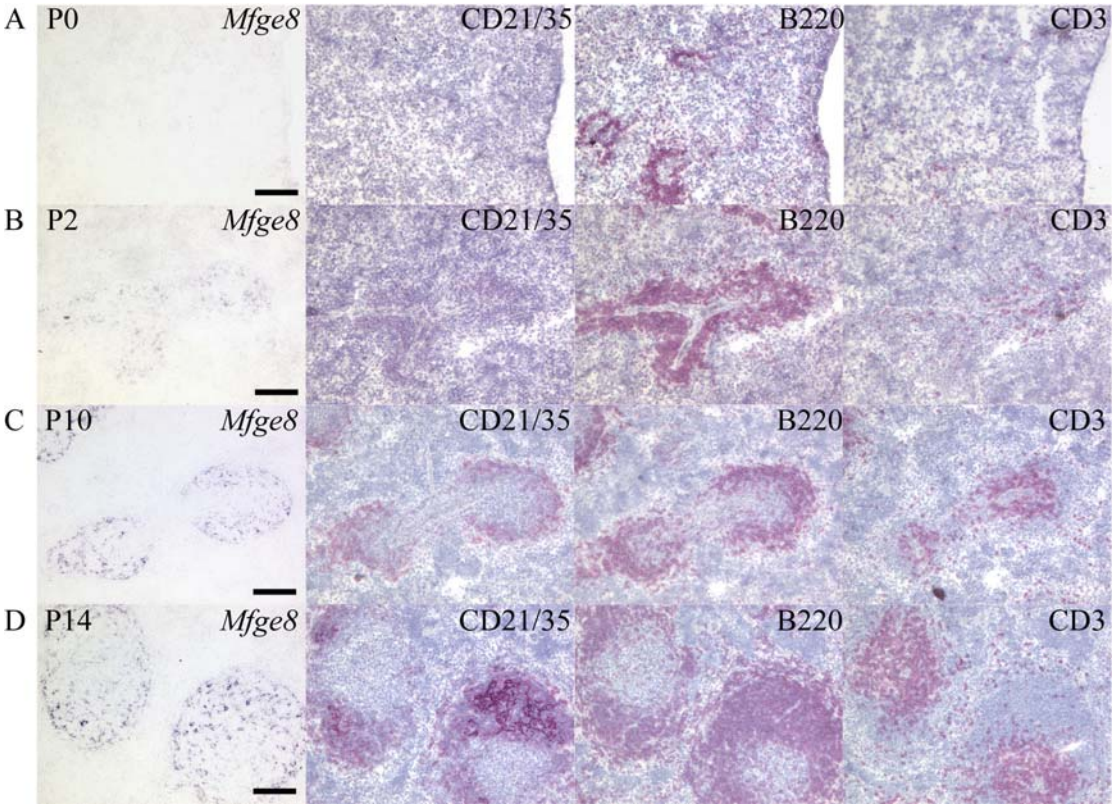


Fig. 23 Development of FDCs in the postnatal spleen
Analysis of postnatal spleens by ISH for *Mfge8,* IHC for CD21/35, B220, and CD3, was performed to assess the development of the hemaopoietic compartment. At postnatal day 0 (P0, A) no Mfge8 expression could be observed. At P2 (B) *Mfge8* expressing cells were detected at perivascular sites, in the presence of lymphocytes expressing B200 and CD3. P10 (C) follicular structures were present and B cells started to express CD21/35, segregation into B and T cell zones were observed. P14 (D) CD21/35 high clusters were detected within the FDC network. Scale bar 100μm

from postnatal day 0 (P0). Consecutive sections were stained with CD21/35 to determine the status of FDC and B cell maturation, B220 and CD3 were used to detect B and T lymphocytes, respectively. At P0 (Fig. 23 A) and P1 (data not shown), no *Mfge8* expressing cells were found, only by P2 *Mfge8*$^{+}$ cells could be detected appearing around vascular sites, co-localizing with entry sites of B and T cells (Fig. 23 B). B cells present in the first postnatal week did not express CD21/35 indicating that they were still not fully mature, but early transitional (T1) B cells (Loder et al.

1999). By P10 follicular structures could be found and CD21/35 was increasingly present, reflecting the more mature state of the B cells present (Fig. 23 C). At P14 FDC clusters with high CD21/35 expression were present within the follicles, B and T cell separation had occurred (Fig. 23 D).

FDCs are derived from a Pdgfrb expressing cell

Previous results pointed to a perivascular origin of the mature FDC: the earliest *Mfge8*$^{+}$ cells were accumulating along vessels, and transitional forms between perivascular, MS lining and follicular *Mfge8* expressing cells were observed. Within the perivascular niche, mesenchymal pericytes were shown to be multipotent cells, which could differentiate into different cell types including fibroblasts (Dellavalle et al. 2007; Lin et al. 2008; Tang et al. 2008). To test for a perivascular origin of FDCs, we used pericyte reporter mice. PDGFRβ is a receptor expressed specifically in

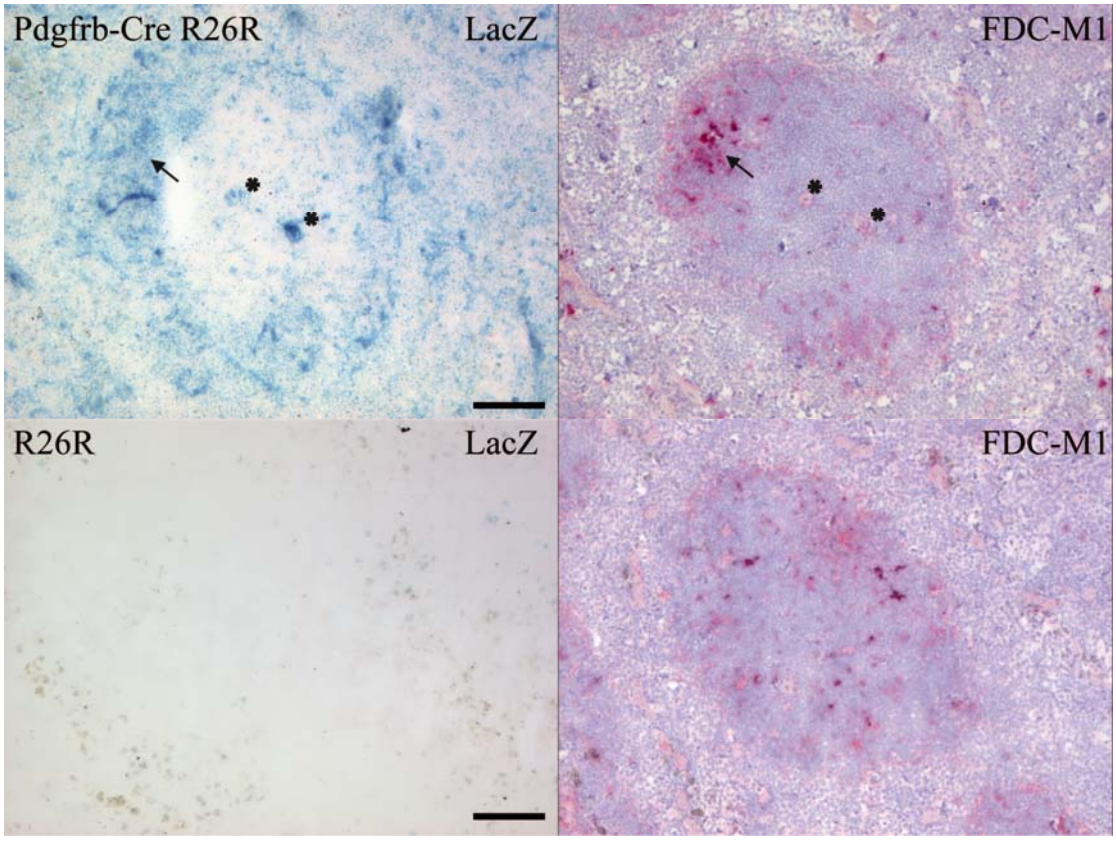


Fig. 24 Using a pericyte reporter mouse to determine the origin of FDCs
Spleens from Pdgfrb-Cre^{+} R26R^{+} (upper row) and Pdgfrb-Cre^{-} R26R^{+} (lower row) littermate (n=1) were stained for β-Gal activity (left), consecutive sections were stained for FDC-M1 (right) do detect FDCs. β-Gal activity was found in the areas of mature FDC networks but also in the MS and the RP. Scale bar 100μm.

pericytes of different organs. We used Pdgfrb-Cre transgenic, crossed to a Cre reporter mouse expressing beta galactosidase (β-Gal) as a result of Cre activity (Fig. 24). Consecutive splenic sections were either stained for β-Gal activity or FDC-M1. The FDC marker co-localized to the same areas as the β-Gal staining did. FDCs express or must have expressed Pdgfrb at some time during their development, suggesting that FDCs are related to pericyte cells or are even of pericyte origin.

preFDCs express pericyte markers

To test whether mature FDCs and preFDCs were maintaining a pericyte character, co-immunofluorescence for the pericyte markers alpha smooth muscle actin (SMA), chondroitin sulfate proteoglycan NG2 as well as PDGFRβ with Mfge8 was performed. Interestingly, mature FDCs showed little expression of any of these markers, while preFDCs in the MS exhibited SMA and PDGFRβ expression, but were negative for NG2 (Fig. 25). This indicates that during early development future FDCs express pericyte markers, but downregulate these during further differentiation into mature FDCs. As pericytes do not always express all the reported markers, this might explain why the NG2 expression in the spleen is present to a much lesser extent than SMA or PDGFRβ are.

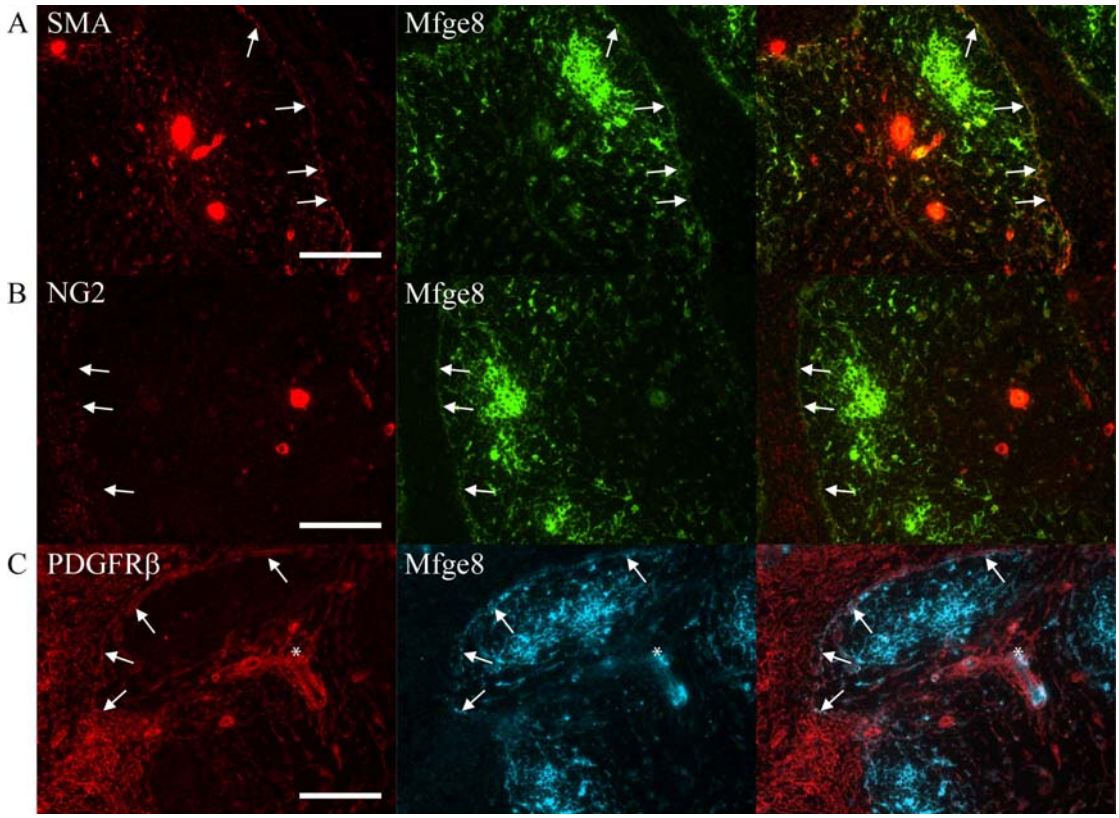


Fig. 25 Expression of pericyte markers in the spleen
Co-immunofluorescence on WT spleens of Mfge8 with the pericyte markers SMA (A), NG2 (B) and PDGFRβ (C) were performed. Mature FDCs showed little expression of pericyte markers, but preFDCs in the MS expressed SMA and PDGFRβ. Arrows point to preFDC with pericyte marker expression. * indicates central arteriole. Scale bar 100μm.

Mfge8 expressing cells in liver and kidney

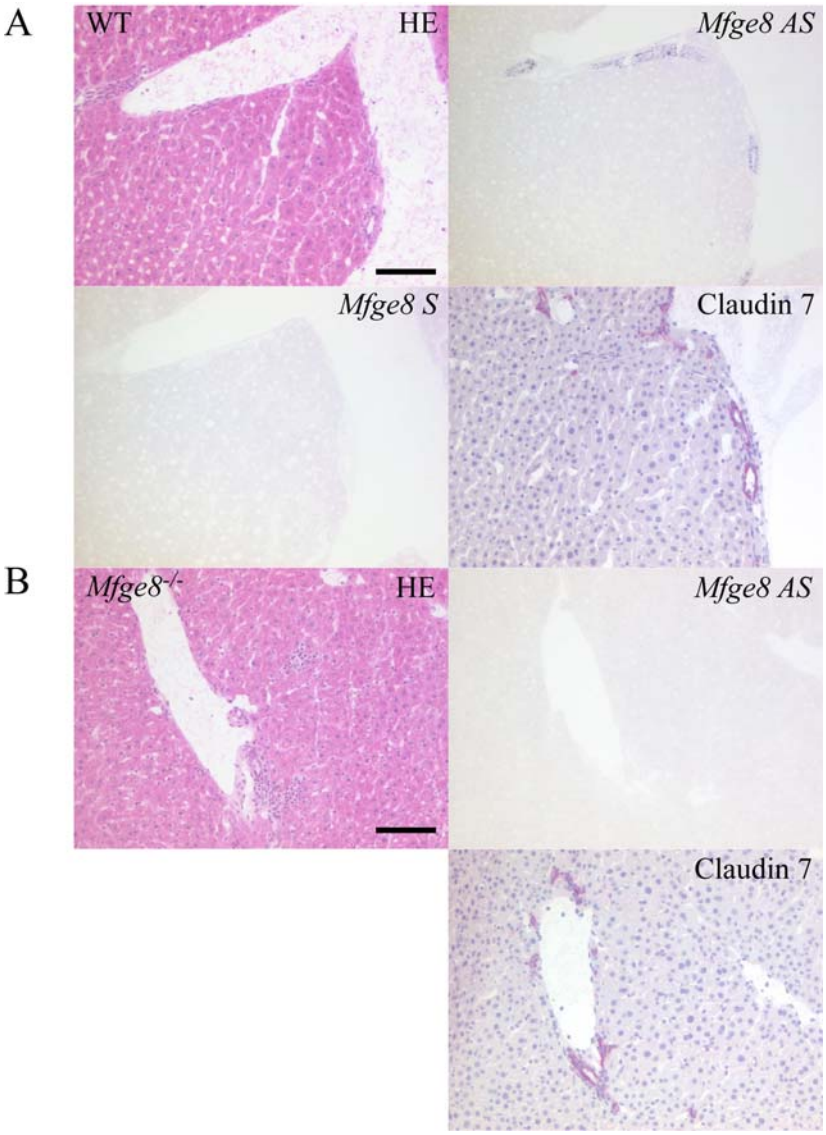


Fig. 26 Expression of *Mfge8* in the WT liver
ISH for Mfge8 was performed on WT livers (A), and *Mfge8*$^{-/-}$ livers (B). Consecutive sections were either hybridized with a negative control sense probe (*Mfge8 S*), stained with HE, or the oval cell marker Claudin 7. Scale bar 100µm.

Because ectopic expression of LT leads to the induction of tertiary lymphoid follicles in non-lymphoid organs, such as liver, and kidney, I was interested to see whether putative proFDCs were present. We wanted to see whether *Mfge8* expressing cells were found *in situ*, whether these depended on LTβR activation, and whether changes in the presence of LTβR activation could be observed. Studying WT livers I realized that Claudin7^{+} oval cells, also know as biliary cells of liver, expressed *Mfge8* (Fig. 26). Interestingly, oval cells expressing *Mfge8*, were also found in *Ltbr*$^{-/-}$, thus steady-state *Mfge8* levels in the liver are independent of *Ltbr*$^{-/-}$ (Fig. 27). As reported these mice have inflammatory foci in their livers containing T and B cells without development of FDCs. Often these inflammatory foci seemed to localize to oval cell areas. Since FDCs were reported to develop in livers upon presence of LT, we tried to simulate an

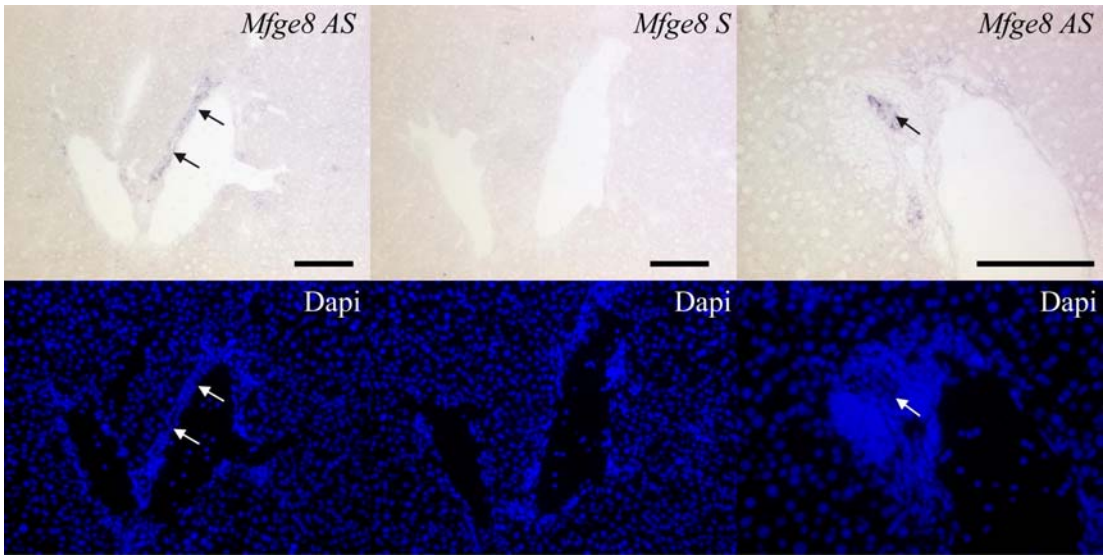


Fig. 27 Expression of Mfge8 in the absence of LTβR activation
ISH for *Mfge8* on liver sections of *Ltbr*$^{-/-}$ mice, counterstained with Dapi. Consecutive sections hybridized with sense probe. Higher magnification picture shows that oval cells next to the portal field are surrounded by lymphocytic infiltrates, typically seen in *Ltbr*$^{-/-}$ mice. Scale bar 100μm.

environment of FDC induction by treating WT mice with agonistic anti-LTβR antibody. We found that compared to isotype control treated mice 24h after i.v. treatment, *Mfge8* was upregulated approximately 9 fold in agonist treated livers. Oval cells showed a strong *Mfge8*$^{+}$ signal, but also cells throughout the liver started to express *Mfge8*, while isotype control treated mice had unaltered expression restricted to oval cells only (Fig. 28). Whether these cells represent already differentiated FDCs or stellate cells, the liver pericytes, which have a dendritic appearance, remains to be shown.

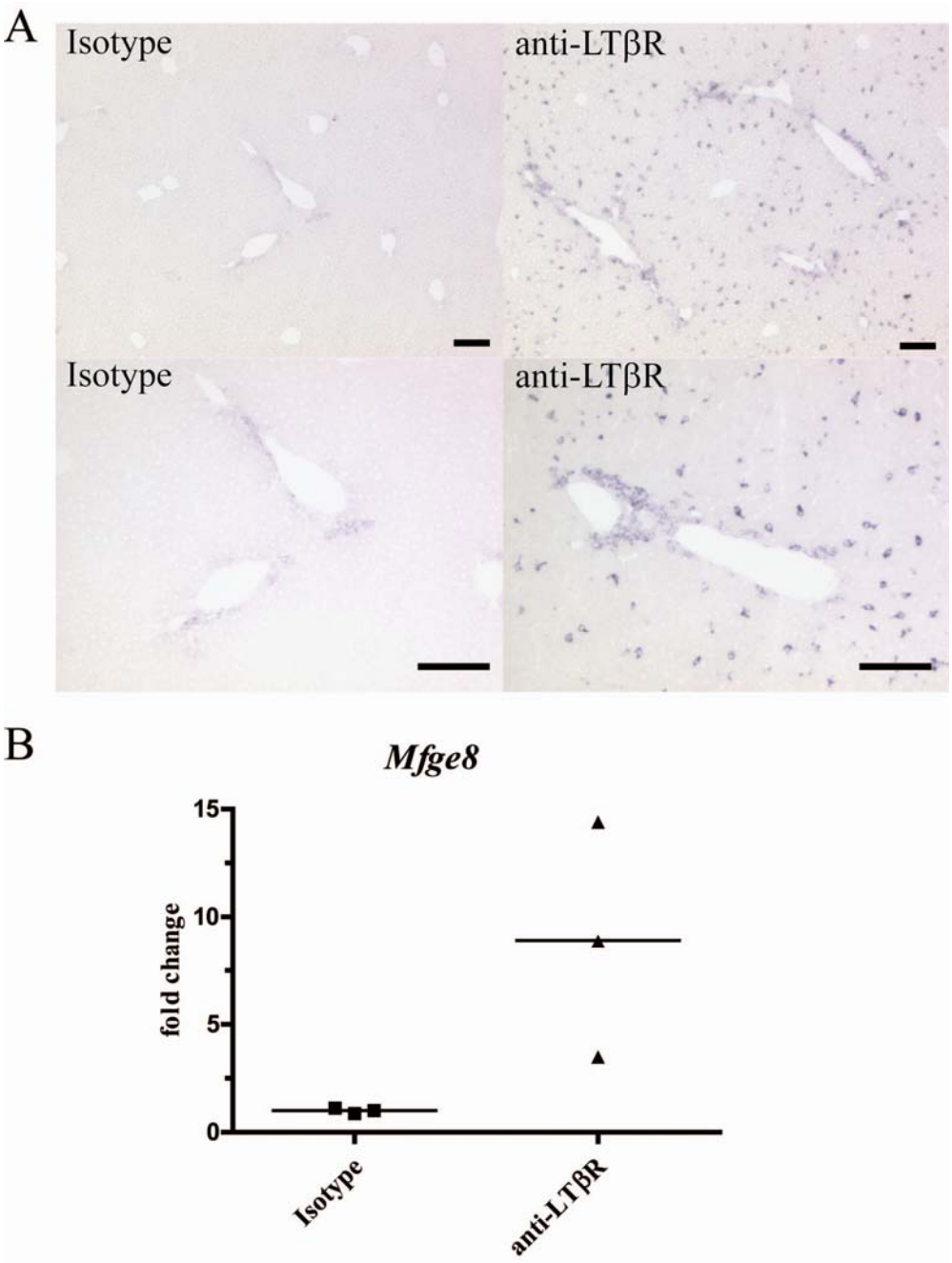


Fig. 28 Induction of *Mfge8* expressing cells in the liver
WT mice were treated for 24h with agonistic anti-LTβR antibody or hamster isotype control (n=3). Livers were analyzed by *Mfge* ISH (A): lower magnification (upper row) and higher magnification (lower row). Scale bar 100µm. The fold change in Mfge8 expression was determined by qPCR, normalized over *Gapdh* and quantified over isotype control. Compared to isotype treated mice (1±0.1 SD) agonist treated mice showed in mean an increase of. (8.9±5.5 SD). SD standard deviation. Statistical analysis using unpaired t-test revealed that the increase was not significant.

Furthermore the presence of *Mfge8* expressing cells in the kidney was examined by ISH (Fig. 29). Indeed, *Mfge8*$^{+}$ cells could be detected present in the glomeruli of the

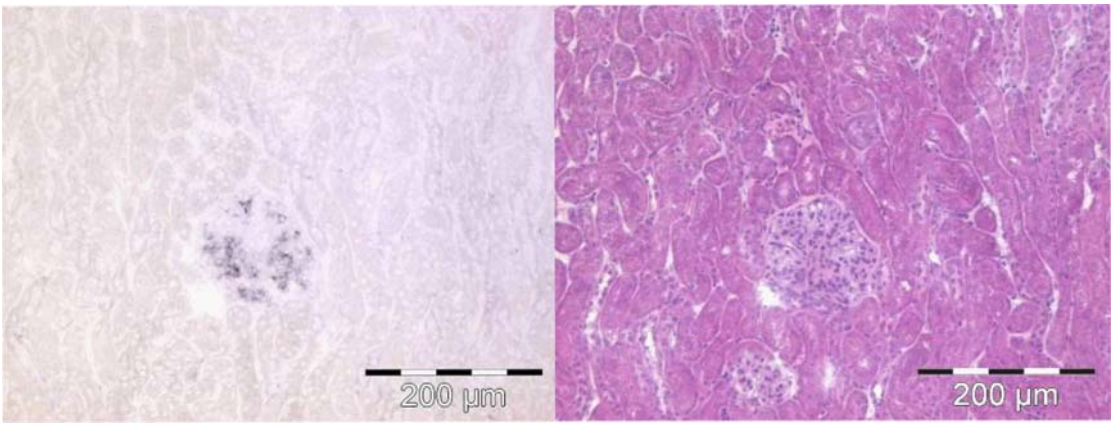


Fig. 29 *Mfge8* expressing cells in the kidney
Kidney of a WT mouse hybridized with an *Mfge8AS* probe (left), consecutive section was stained with HE (right). Mfge8 RNA is detected within the glomeruli of kidney and might be expressed by mesangial cells

kidney. The mesangial cells, which are specialized pericytes of the kidney, reside within the glomerulum, suggesting that pericytes in different organs might express *Mfge8*.

GENERATION OF BAC TRANSGENIC MICE

Cloning strategy of BAC transgenic mice

Several functions have been ascribed to FDCs. Most of them remain sketchy, as there are no methods for culturing FDCs in a pure state, and due to a dearth in molecular FDC-specific markers, there are no means of targeting FDCs transgenically. We have generated first evidence that FDCs are developing from a perivascular pericyte expressing Pdgfrb, and that Mfge8 is an early marker for these cells. We have, however, no formal proof that early *Mfge8* expressing cells in spleen and other organs can (and will) develop into mature FDCs. Fate tracing by use of inducible reporter mice can be performed to answer this question. To evaluate FDCs' involvement in the establishment of chronic inflammations, or cancer, and their supposedly beneficial role in the prevention of autoimmunity, a conditional ablation and genetic targeting of FDCs would be advantageous. Also, FDCs play a major role in prion diseases. Presence of prions in the spleen and neuroinvasion of prions into the CNS is dependent on FDCs. However, whether FDCs indeed replicate PrP^{Sc} or merely accumulate it, remains to be answered.

Therefore, we decided to generate mice expressing different genes of interest (GOI) under the Mfge8 promoter. The GOIs are Cre recombinase, tamoxifen-inducible Cre recombinase, Prnp, tdTomato, Lta, and Ltb.

Cre (Cyclization Recombination) recombinase is a viral recombinase derived from the bacteriophage P1. The Cre recombinase recognizes loxP sites – a loxP site is a 34bp DNA sequence containing two palindromic areas. Cre-mediated recombination of two loxP sites can be used to delete loxP flanked areas (Gu et al. 1993). In transgenic approaches the Cre recombinase can be expressed under a specific promoter, a technique, which is frequently used to generate conditional knock outs or to restrict a reporter expression to a certain tissue or cell type, which allows the tracing of cell populations (Lakso et al. 1992; Gu et al. 1994).

Inducible Cre-recombinase ($CreER^{T2}$) is a fusion of Cre recombinase to a mutated estrogen receptor (ER). ER^{T2} has a high affinity for synthetic tamoxifen, but does not bind estradiol (Feil et al. 1997). $CreER^{T2}$ also lacks the endogenous nuclear localization signal (NLS). In the absence of the NLS the recombinase remains in the cytoplasma, however, hormone-binding results in the nuclear translocation of fusion

protein, where Cre recombinase exerts its function, namely the recombination of loxP sites. Thus the expression of $CreER^{T2}$ under the Mfge8 promoter allows the tamoxifen-inducible recombination of LoxP sites with a restriction to $Mfge8^{+}$ cells, making it a useful tool for fate tracing of FDCs.

Restricting the PrP-expression to FDCs by placing the Prnp gene under the Mfge8 promoter, will allow us to tackle the question of FDC-intrinsic prion replication capacity.

tdTomato (tandem dimer), is a strong red fluorescent protein, which will be used to follow cells expressing Mfge8 *in vivo* (Shaner et al. 2008). Fluorescently labeled cells will be isolated and cultured *in vitro* or used for transplantation. The Mfge8-tdTomato mouse strain will facilitate understanding cellular interactions and signaling mechanism behind FDC development.

Expression of LTα and LTβ under the Mfge8 promoter will be used to investigate whether early FDCs ($Mfge8^{+}$ cells) can stimulate innate LTβR signaling in an autocrine or paracrine fashion to generate mature FDCs ectopically in organs such as the liver or kidney.

As very little is known about the promoter and enhancer areas of Mfge8, we decided to generate transgenic mice using Bacterial Artificial Chromosomes (BAC), which contain long DNA fragments including the Mfge8 locus and all its regulatory regions (Yang et al. 1997). BACs are circular DNA vectors, which enable the propagation of large foreign DNA fragments in Escherichia coli (E.coli). The corresponding DNA fragments are inserted into a fertility plasmid, which allow the

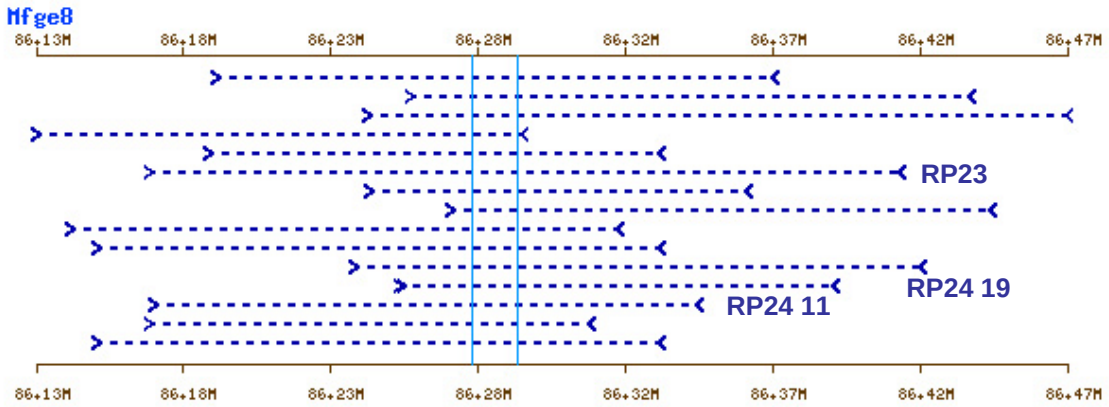


Fig. 30 Different BACs containing the Mfge8 locus
NCBI database was searched using clone finder for BACs containing the Mfge8 locus (blue dotted lines each represent the fragment of each single BAC). Three different clones used for the generation of BAC transgenics are indicated: RP23, RP24 19, and RP24 11.

equal distribution of the vector after replication to the daughter cells. DNA fragments used to generate BACs usually have a size of 150-350 kilo bases (kb).

The use of BAC-based transgenesis usually eliminates the problem of position effects that occur in classical transgenic mice. Furthermore as DNA fragments contain all the regulatory elements of the GOI, this usually leads to predictable transgene expression patterns.

To find BAC clones, which contain DNA fragments including the Mfge8 locus (chromosome 7, 41.2 cM), we searched the national center of biotechnology information (NCBI) using their software clone finder. We found several clones containing the Mfge8 locus, but chose RP23-24N16 (BAC23), a clone containing a 247kb sized genomic insert, which had been generated by insertion of a partial EcoRI digestion of C57Bl6 mouse genomic DNA and subsequent ligation with the BAC back backbone. RP24-246M19 (BAC24 19), and RP24-378M11 (BAC 24 11), were kept as alternative clones.

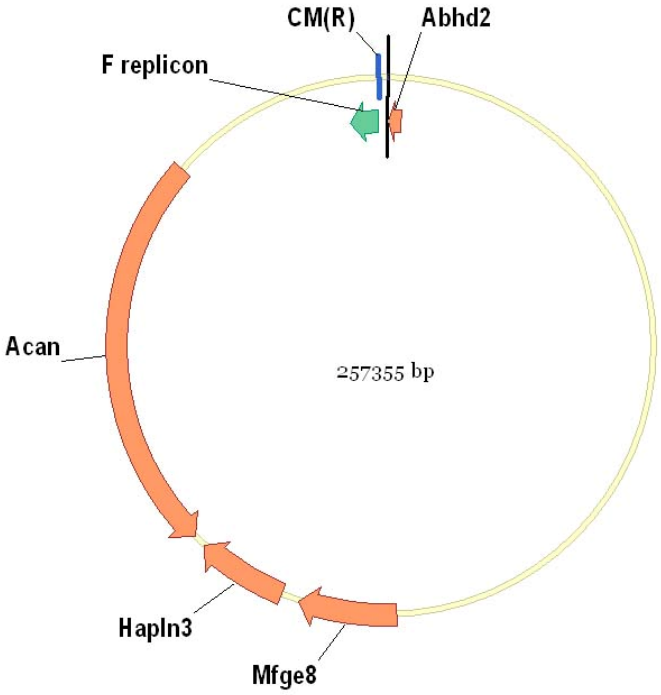


Fig. 31 Schematic depiction of the BAC RP23
BAC backbone with a 247kb insert of mouse genomic DNA containing the Mfge8 locus. Genes are drawn in orange, arrow head points to the 3'end of the gene. CM(R) indicates the chloramphenicol resistance .F replicon (F plasmid). Picture made in VNTI 10 (Invitrogen).

In BAC 23, the Mfge8 gene (15kb length) is localizing in the central area of the insert, ensuring that all regulatory elements are present (Fig. 30 and Fig. 31). The disadvantage of BAC technology is, that the big genomic fragments used to generate transgenics, frequently contain additional genes apart from the GOI. The generation of BAC transgenic mice might therefore result in their co-expression and generate unexpected phenotypes. BAC 23 contains the gene encoding for aggrecan (Acan), an extracellular matrix protein involved in axonal outgrowth, Hapln3

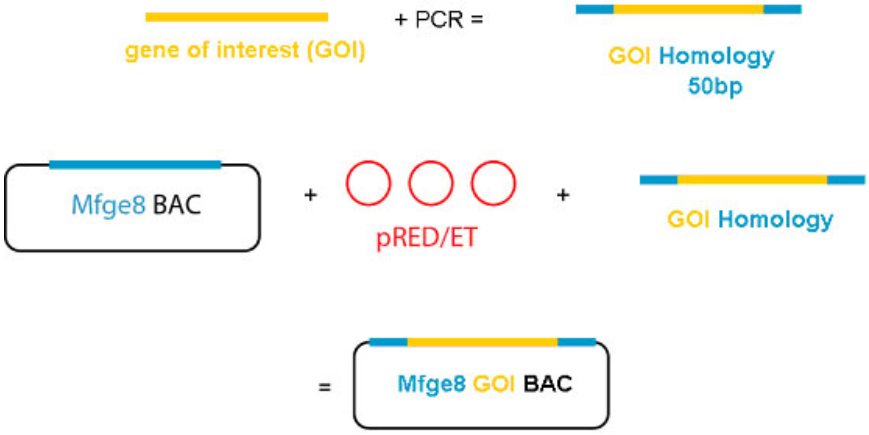


Fig. 32 Scheme of events during homologous recombination
Step 1 (upper row): the targeting cassette, containing the GOI as well as an antibiotic resistance gene, is added flanking homology arms of 50bp size by PCR. Step 2 (middle row): E.coli containing the BAC are transformed with a plasmid for the RED recombinases as well as with PCR product. BAC after undergoing targeted recombination into the Mfge8 locus (lowest row)

(hyaluronan and proteoglycan link protein 3), a protein with so far unknown function, as well as the first 2kb of Abhd2 (abhydrolase domain containing 2), containing only parts of the non-coding sequence (Fig. 31).

Due to their large size BACs contain a multitude of restriction sites, making a restriction digest approach to insert the GOI into the BACs impossible. Instead homologous recombination is the technique to insert a GOI at a site of interest in bigger plasmids (Fig. 32) (Johansson et al. ; Zhang et al. 1998). To enable homologous recombination, PCR based technique is used to flank the GOI with 50bp 5' and 3' homology arms that are complementary to the target area on the BAC fragment. The PCR product and a plasmid encoding for the RED proteins essential for recombination are transformed into the E.coli containing the BAC. The RED proteins are Redα, which functions as a 5'-3' exonuclease, and Redβ, a single strand binding protein. In their presence the ends of the GOI-PCR product become single stranded DNA and undergo homologous recombination into the target site of the BAC. As the targeting cassette also contains an antibiotic resistance gene, BACs undergone recombination can be selected by growing E.coli in the presence respective antibiotic.

We planned to insert the targeting cassette at the translational start of Mfge8, at position 93bp in exon 1 and replacing the remaining exon 1, but leaving the other introns and exons unchanged (Fig 33). The targeting cassette consists of the GOI, a 3' polyadenylation (polyA) sequence followed by the antibiotic resistance gene neomycin (NeoR). Furthermore, we replaced the Mfge8 translation start site (AGCATG) by the by the classical Kozak consensus sequence (ACCATG), this sequence is known to facilitate optimal translation initiation.

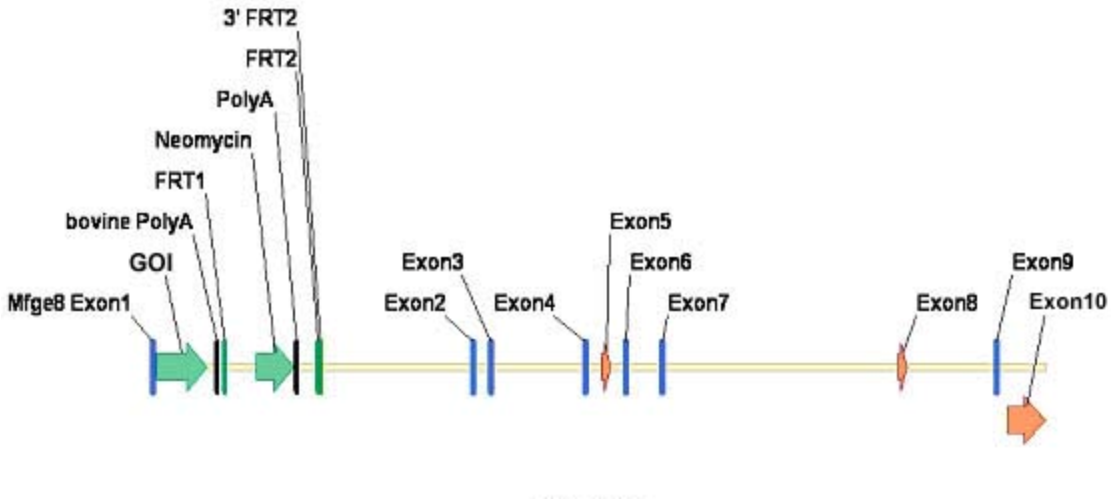


Fig. 33 Scheme of the targeted Mfge8 locus
The targeting cassette (in green) is inserted into the Mfge8 locus at exon 1 replacing the endogenous Mfge8 ATG at position 94 amino acids (aa). The targeting cassette contains the GOI, followed by the bovine sequence needed to add the Poly A tail, needed for stabilization and translation of the generated mRNA. NeoR is flanked by Flp recognition target sequences (FRT).

Verification of the BAC clones

E. coli clones transformed with one of the three different BAC clones, BAC23, BAC 24 19, or BAC24 11, were obtained from BACPAC resource center. The backbone of

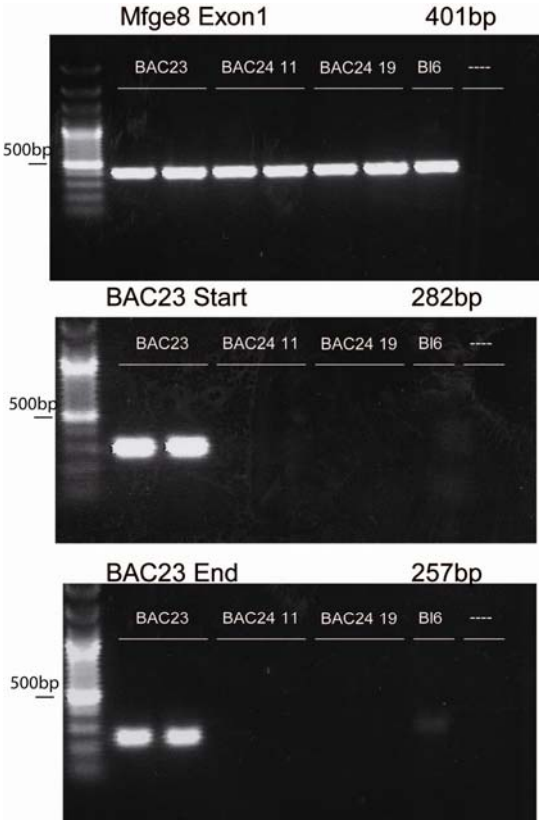


Fig. 34 First verification of BAC 23
Two clones of BAC23, BAC24 19 and BAC24 11, each, were tested for the presence of Mfge8 exon 1 (upper row) using flanking primers (401bp), Bl6 (WT mouse tail) as positive control, ---- water control. Insertion genomic DNA into the backbone was confirmed performing PCRs on the backbone/insertion intersection. BAC23 insertion start (BAC23 start 282bp (middle row)) and BAC 23 insertion end (BAC 23 end 257bp (lowest row))

these constructs contained origin of replication and a chloramphenicol resistance gene (ChlorR). For each BAC, 2 subclones were picked from a chloramphenicol (Chlor) LB agar plate and expanded and PCRs performed to verify the clones. The presence of the Mfge8 exon 1 locus was first verified by PCR (Fig. 25). All BACs contained the Mfge8 exon 1 locus. Compared to the other clones BAC 23 should contain the biggest genomic insert and thus all regulatory elements of Mfge8. The other advantage of BAC 23 over the other clones is that the insert of BAC 23 had been fully sequenced and published. BAC 23 was therefore chosen to proceed with for targeting. To verify that the purchased BAC 23 not only contained the entire 250kb insert, PCRs with primers covering the intersection area between BAC insert and backbone were performed (BAC 23 start and end) (Fig. 34).

Cloning plasmids for the generation of targeting constructs

For the generation of the different Mfge8 constructs, Cre (CrepANeofrt) and CreER^{T2} (CreERT2pANeofrt) plasmids, with a 3' polyA and a FRT flanked NeoR were obtained from Dr.Thorsten Buch and verified by sequencing (Fig. 35). FRTs (Flp recognition target) allow flippase (Flp)-mediated recombination of these sites and excision of the flanked area. Plasmids containing Prnp, Lta, Ltb and Tomato ORFs were sequenced and then subcloned into the CrepANeofrt to add the polyA and the FRT NeoR (Klein et al. 1998; Shaner et al. 2004; Heikenwalder et al. 2005). Subcloning was achieved by enzymatic cleavage and ligation, replacing the full sequence or parts of the Cre. The CrepANeofrt plasmid has single cut restriction site

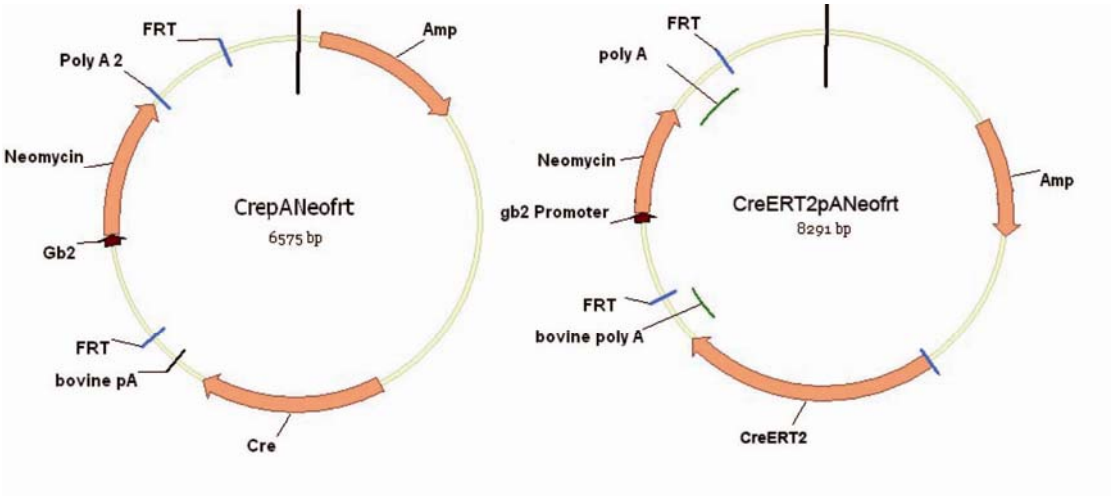


Fig. 35 Plasmids for the generation of Mfge8-Cre and Mfge8-CreERT2
Scheme of the plasmids obtained for the generation of Mfge8-Cre and Mfge8-Cre ERT2 targeting cassettes. They contain Cre (left), and CreERT2 (right), respectively, with a 3' bovine Poly A, and an FRT flanked NeoR (Neomycin), with a prokaryotic promoter (gb2) and a 3' PolyA. Amp: ampicillin resistance gene

MluI at the end of the Cre gene, 5' of the bovine polyA, as well as a single cut restriction site within the Cre gene, BstbI (=SfuI), and another motif recognized by KpnI 5' of Cre gene. As the sequence for Lta and Prnp, already contained a KpnI cleavage, the combination BstbI and MluI was used as subcloning strategy. Ltb and tdTomato were inserted by KpnI/MluI digestion and ligation with the CrepANeofrt plasmid (Fig. 36).

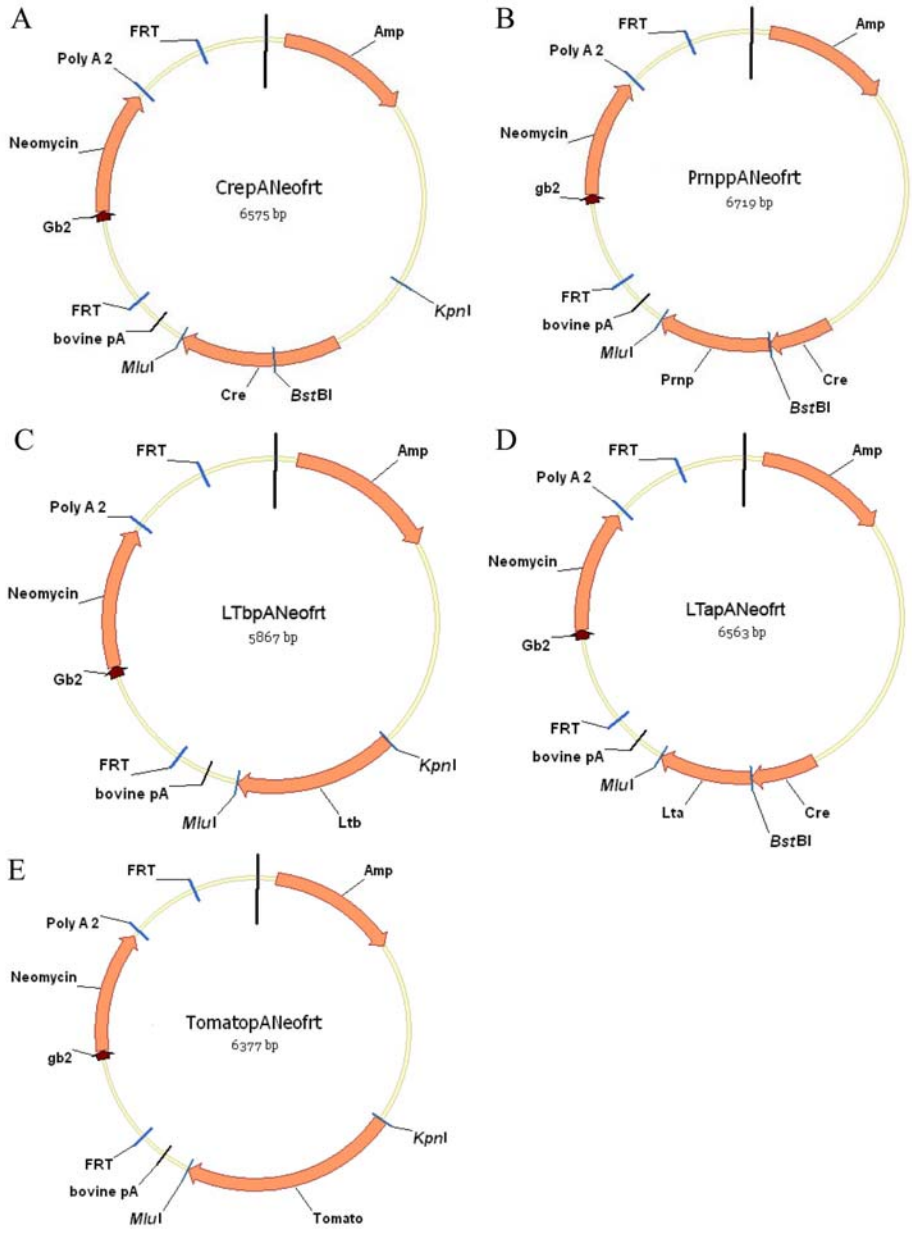


Fig. 36 Scheme of plasmids cloned to be used for the generation of the targeting cassette
Substitution of Cre gene from the CrepANeofrt plasmid (A) by Prnp (B), Ltb (C), Lta (D), Tomato (E) using single cutting restriction sites, KpnI, BstbI, and MluI. Lta and Prnp are inserted using BstbI, as their sequence contains internal KpnI cutting site. Gb2 or gb2 prokaryotic promoter, FRT (Flp recognition target), Amp ampicillin resistance gene, Neomycin NeoR.

The respective restriction motifs were added to the GOIs by PCR (Fig. 37 A). PCR products were cut from the gel and purified, the DNA content determined and the fragments digested with the respective pair of restriction enzymes. CrepANeofrt was digested with either combination of restriction enzymes and the backbone gel purified (Fig. 37 B). Inserts and backbone were ligated and transformed into E.coli, which were grown in ampicillin to select for ligated constructs. Single clones were expanded and proper insertion verified by sequencing.

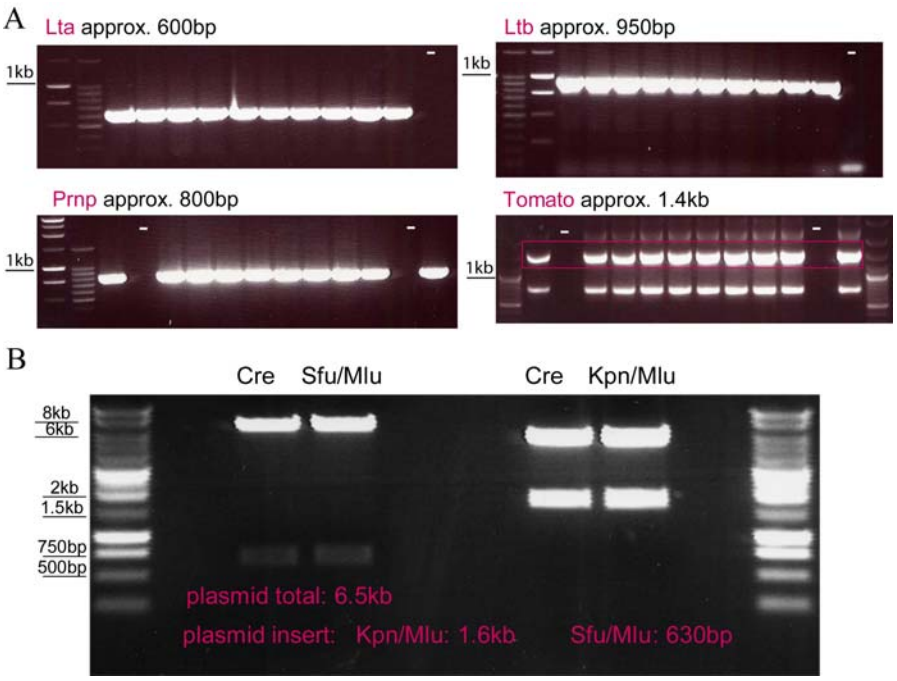


Fig. 37 Cloning Lta, Ltb, Prnp, and tdTomato into CrepANeofrt plasmid
Addition of BstbI/MluI restriction motives to Lta and Prnp, as well as KpnI/MluI motives to tdTomato, Ltb by PCR. Separation of PCR products by electrophoresis, DNA size markers (lane 1 and 2), (-) PCR without DNA (A). Tomato showed three amplified products, with the main band running at the estimated size of 1.4kb. (B) CrepANeofrt digested either with KpnI/MluI (3rd, 4th lane) or SfuI/MluI (7th,8th lane), and run over agarose gel, shows the digested backbone and insert.

Generation of the targeting cassettes

To generate the targeting cassette for homologous recombination primers were designed that they contain 50bp homology arms with the insertion region as well as 25bp overlapping with the GOI. Only high quality, PAGE purified primers were used for the addition of homology arms. For the PCR, high fidelity and high speed DNA polymerases were employed. For all constructs except for Ltb, homology arms could be added. PCR products were gel purified and proceeded with for the homologous recombination (Fig. 38).

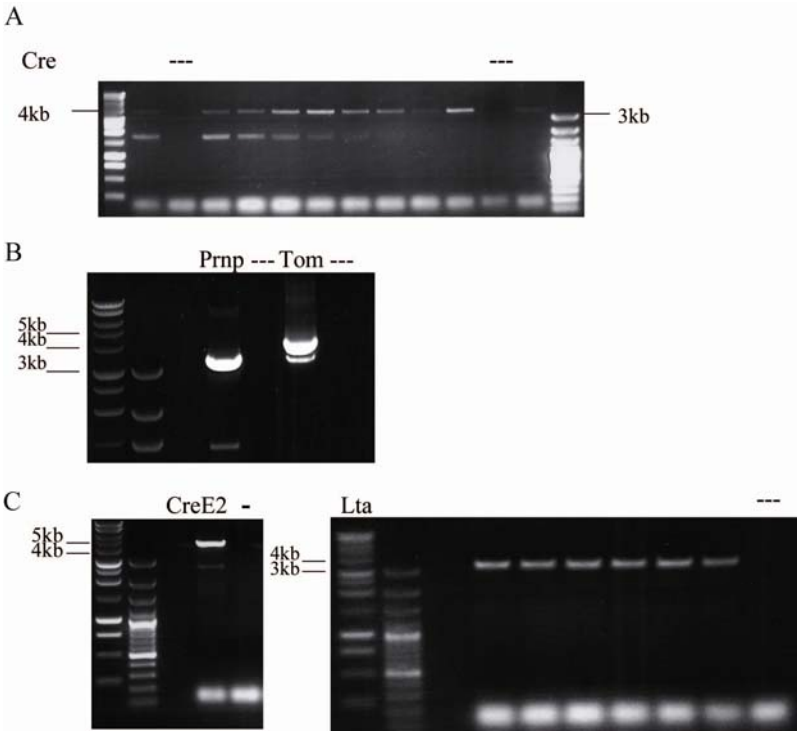


Fig. 38 Addition of homology arms
Targeting cassettes for the Mfge8 locus were generated by PCR amplification using primers containing 50bp of homology arms and 25bp of annealing sequence of the specific insert containing the GOI as well as the FRT flanked neomycin. (A) Cre targeting cassette (3.7kb, upper bad), (B) Prnp targeting cassette (3.2kb, left), and tdTomato targeting cassette (3.9kb, right), (C) CreERT2 (4.4kb, left) and Lta containing cassette with homology arms (3.2kb, right). --- water control.

Homologous recombination

For homologous recombination, BAC23 E. coli were first electroporated with the plasmid encoding for the Redα/Redβ proteins and containing a tetracycline resistance gene (TetR) (Red/ET), and a thermosensitive origin of replication. (below 30°C). The expression of Redα/Redβ mRNA is regulated by an L-arabinose-inducible promoter. E.coli containing the BAC and Red/ET were grown in the presence of L-arabinose before the constructs containing the homology arms were electroporated. During a further incubation step of 1-2h at 37°C, homologous recombination occurred, while the Red/ET plasmid was lost. The NeoR contained within the targeting cassette conferred antibiotic resistance for kanamycin (Kana). After 1-2h E. coli were positively selected for recombination on LB-agar plates containing Kana, single clones picked and tested for their insertion by PCR (Fig. 39; and data not shown). The absence of non-recombined BAC was further confirmed by PCR amplifying the

original Mfge8 exon1 locus (Fig. 40). 3 clones had inserted Cre, 2 tdTomato, 1 Lta, 3 Prnp and 9 CreERT2 into the Mfge8 exon 1 locus.

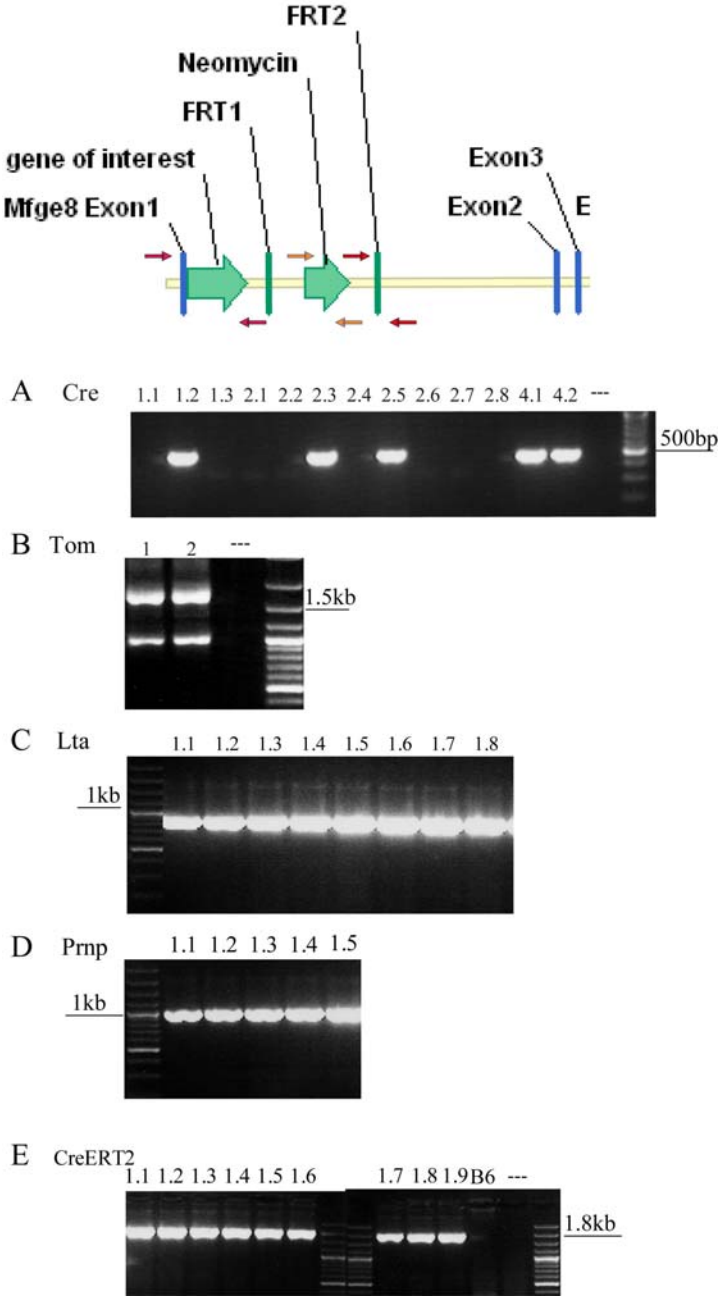


Fig. 39 PCR to test for insertion of targeting cassette
To test the insertion of the targeting cassette into the Mfge8 exon 1 locus different PCRs were performed. Primer pairs used are indicated in the scheme in different colours. PCRs with primers annealing at the intersection of the 5' region of the insertion locus were used to verify the recombination of the targeting cassette into the Mfge8 exon 1 locus. PCRs for the NeoR gene and the insertion end area are not shown (A) insertion of Cre (461bp) change picture in 5/13 clones, (B) tdTomato (1.8kb), (C) Lta (900bp) numbering of picture, (D) Prnp (1.1kb), (E) CreERT2 (2.1kb). B6 (WT) and (---) water as negative controls.

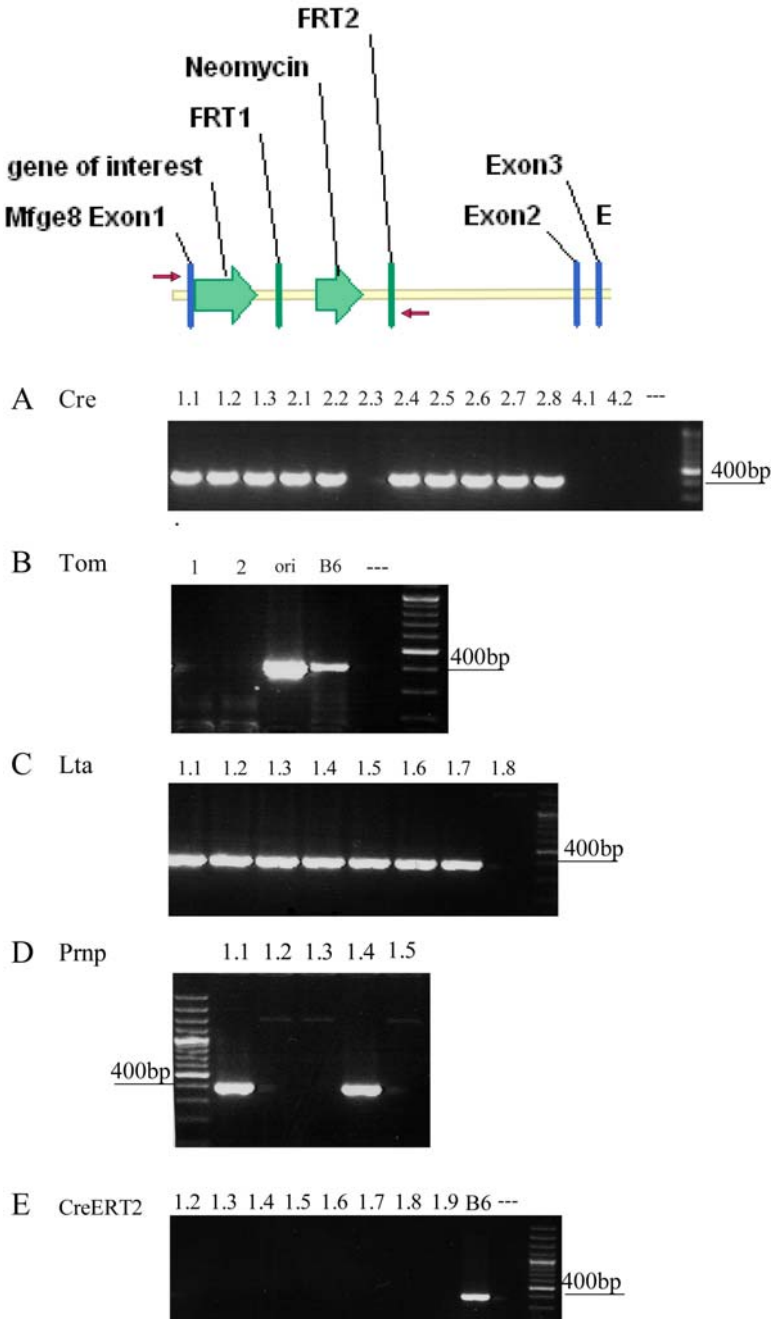


Fig. 40 PCR to test for the removal of the endogenous Mfge8 Exon 1 locus
PCRs with primers annealing upstream and downstream of the Mfge8 Exon1 locus were used to test whether the endogenous Exon 1 had been ablated during the targeting event (401bp). Though these primers still anneal, 5' and 3' of the targeting cassette, the segment to be amplified is too large to be amplified in this PCR. Clones that remain positive for Mfge8 exon 1 PCR have a contamination with non recombined BAC constructs (A) Cre targeting, clones 2.3, 4.1, 4.2 had undergone recombination, and no contamination with non-recombined BAC (B) Both tdTomato clones are negative for the endogenous Mfge8 Exon 1 (C) Lta clone 1.8 had lost the endogenous Mfge8 (D) Prnp clones 1.2, 1.3, 1.5 had recombined properly (E) CreERT2 all clones had undergone insertion into the Mfge8 locus. Unrecombined BAC (ori), B6 (WT mouse tail DNA) positive control. --- water control.

Removal of the neomcycin resistance gene

The targeting cassette of our constructs not only contained a prokaryotic promoter site (gb2) 5' of the NeoR gene to allow the selection of BACs having undergone homologous recombination, but also the strong eukaryotic phosphoglucokinase promoter (pGK), which allows expression of the constructs in vitro, by transfection of mammalian cells and selection for NeoR. However, in transgenic mice, the presence of the pGK promoter in proximity to the GOI's promoter, can lead to competition for transcription factors, resulting in aberrant GOI expression. We, therefore, intended to ablate the pGK/gb2 promoter and the NeoR, by recombination of the FRT sites flanking this area. For this purpose the 706-Flp-tet plasmid (Genebridges) was used. 706-Flp-tet is a low copy number plasmid, whose replication is thermosensitive - thus the plasmid can be propagated below 30°C, but is lost at higher temperatures. It also contains a TetR, and a heat-inducible promoter (37°C), driving the transcription of Flp only at 37°C (Cox 1983). These characteristics allow the recombination of FRT sites, and at the same time guarantee the loss of the plasmid after the recombination event. E.coli containing the recombined BACs were electroporated with 706-Flp-tet, and selected for TetR. Single clones were picked and heat-induced over night in LB medium to allow recombination of FRT sites. Same amounts of cells were plated on a LB agar plate containing Neo/Chlor or Chlor only, to determine recombination frequency. The increase of E.coli colonies on plates containing Chlor only, indicated that NeoR had been successfully removed. For each targeted BAC, several clones were picked and expanded, and then split 1:1 and grown either in Chlor or Chlor and Neo. Clones which were unable to grow in Neo were tested for their loss of NeoR by PCR (Fig. 41). Furthermore, PCRs were performed to confirm that the GOIs had remained inserted at the insertion site (data not shown).

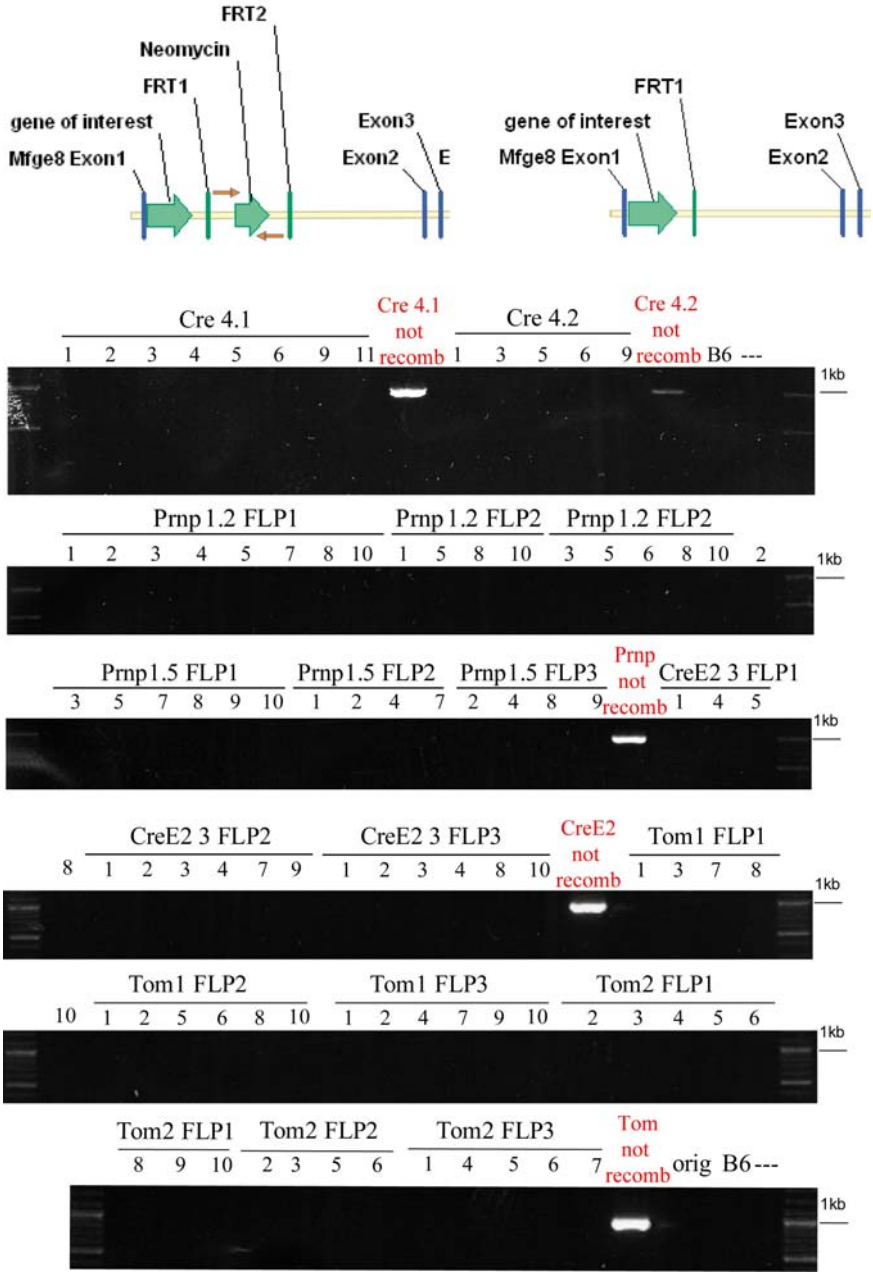


Fig. 41 PCR to test for the ablation of the NeoR gene
Scheme indicating the annealing of NeoR primers before the recombination of FRT sites (left), after recombination (right) annealing sites are ablated. Clones, which were unable to grow in neomycin containing medium were verified by PCR for the loss of NeoR gene. In case of Prnp (1.2, 1.5), CreERT2 (CreE2 1.3) and tdTomato (1,2) each 3 FLP transformed plasmids were grown in presence or absence of Neo. Clones which had lost their ability to grow in presence of neo were tested by PCR for the loss of NeoR, not recombined clones were used as positive control, B6 (WT mouse genomic DNA) and --- water as negative controls.

Though Mfge8-Lta had undergone Flp-mediated recombination of the FRT sites (data not shown), we did not continue with the final verification steps. This was due to the fact, that Mfge8-Lta was meant to be co-expressed with Ltb driven by the Mfge8 promoter, as stimulation of the LTβR is mediated by the LTαβ heterotrimer. However, the generation of the targeting cassette for Ltb had so far failed. We therefore focused on the completion of the Mfge8-Cre, Mfge8-CreERT2, Mfge8-tdTomato, and Mfge8 Prnp. Clones, where the NeoR had been successfully removed. As BACs have a tendency to recombine while being propagated in E.coli, and any changes in the 250kb might alter the expression pattern of our GOIs. To test the integrity of the BACs, DNA from multiple clones/construct was purified and subjected to restriction fragment analysis (RFA). Using enzymes which cut the BAC 20-30 times, this resulted in DNA fragments of 1-50kb size. Fragments were separated by gel electrophoresis, the restriction pattern was compared to the original BAC clone, and verified that the obtained fragments matched the *in silico* size prediction (Fig. 42). RFA confirmed that certain BACs had undergone unwanted recombination events, these BACs were excluded from further analysis.

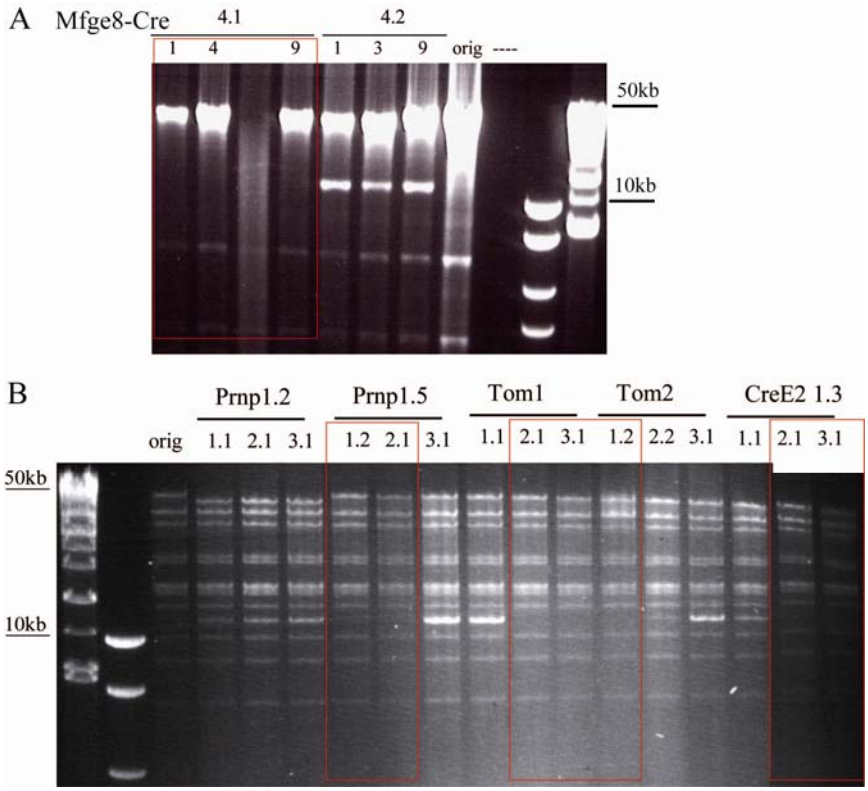


Fig. 42 RFA of BAC constructs
(A) Mfge8-Cre clones were digested with BssHII and restriction fragments compared to the original BAC. All Mfge8-Cre 4.2 clones, had an additional band at 12kb, suggesting that this clone had undergone additional unwanted recombination, clones 4.1 had retained integrity (marked with red box). (B) RFA analysis of Mfge8-Prnp, Mfge8-tdTomato and Mfge8-CreERT2 clones compared to original BAC by XmaI digest. Clones, which maintained integrity, are shown in red box.

Isolation and final verification of the DNA

There are two main requirements for the DNA used for pronuclear injection into the oocyte: right quantity and high quality - there should be little contaminants, meaning other proteins or endotoxins derived from the E.coli, and little DNA degradation. Because BAC DNA is so large, the DNA is less stable than a small plasmid and can easily be sheared into small pieces. Special care has to be taken, and therefore, large orifice pipette tips were used during DNA preparation to reduce shearing forces.

DNA maxipreps were prepared using Sigma Phase prep BAC DNA isolation kit, which allows the isolation of large DNA plasmids and includes repeated endotoxin removal steps. Maxipreps were prepared from Mfge8-Cre (3 clones 4.1.1,

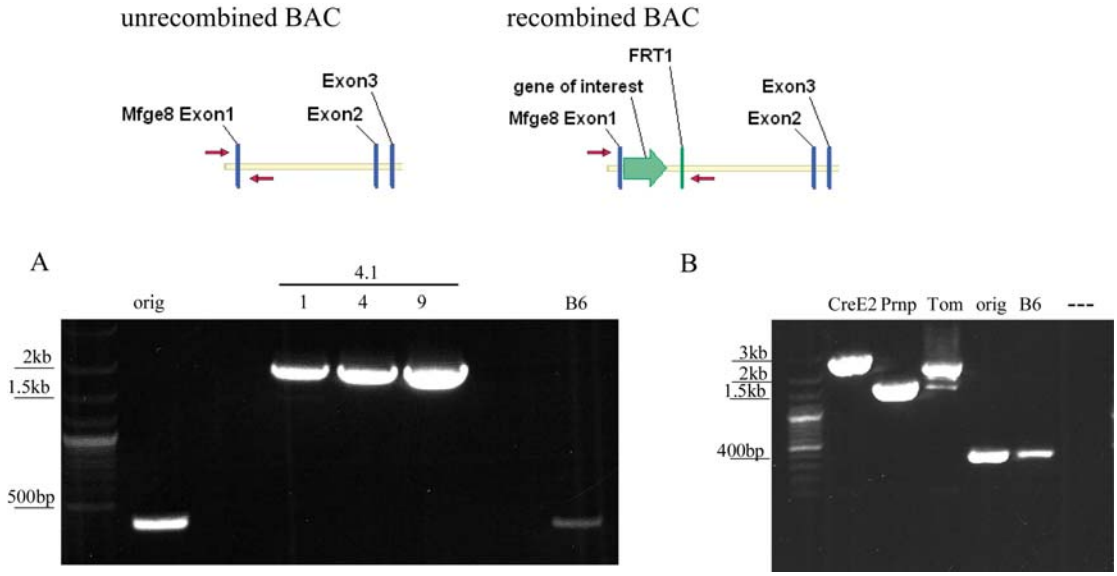


Fig. 43 Final verification of the GOI insertion into the BAC by PCR
Primer pairs were used amplifying the area between Mfge8 exon 1 and intron 1 as indicated in the scheme, thus amplifying the Mfge8 locus of an unrecombined BAC (400bp), as well as of a recombined BAC, which has undergone FLP-mediated removal of the NeoR cassette. (A) PCR run on Mfge8-Cre clones 4.1.1-4.1.9 (1.8kb) and (B) on Mfge8-CreERT2 (2.7kb), Mfge8-Prnp (1.6kb), Mfge8-tdTomato (2.2kb). Original, unrecombined BAC and B6 (WT genomic DNA) served as positive controls for the endogenous PCR. --- contained no DNA All recombined BACs contained fragments of the right size and no contaminating unmodified Mfge8 exon 1

4.1.4, 4.1.9), Mfge8-Prnp (clone 1.5.1.2), Mfge8-tdTomato (clone 1.2.1), and Mfge8-CreERT2 (clone 1.3.2.1). Isolated DNA was resuspended in injection buffer, containing the DNA stabilizing polyamines spermine and spermidine. A final verification of the constructs was done by PCR, sequencing and RFA (Fig. 43, 44, 45). For the PCR primers were used, which anneal upstream of the Mfge8 exon1 as

well as in the intron 1 of Mfge8, thereby amplifying the inserted construct approximately 100bp of the targeted area (Fig. 43). The PCR product was then isolated and sequenced. No mutations were found in Mfge8-Cre, Mfge8-Prnp, Mfge8-CreERT2, Mfge8-tdTomato had an additional cytosine nucleotide in the intron 1 – however this should not result in an alteration of the expression, as it is positioned some hundred bp 3' of the PolyA sequence. Detailed RFA with 2 different enzymes, SpeI and XmaI, was performed to verify the modification and the BAC integrity in the different constructs (Fig. 44, 45).

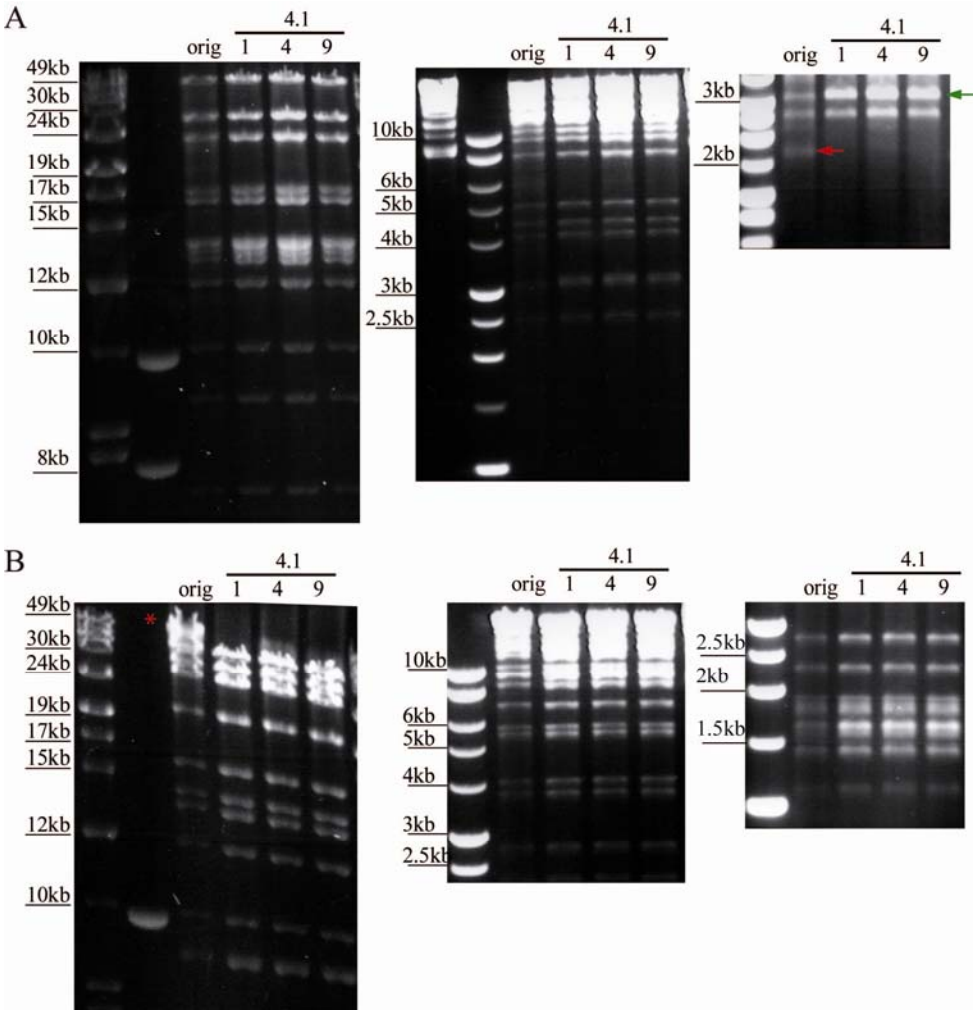


Fig. 44 Confirmation of Mfge8-Cre BAC integrity and modification by RFA
2ug of Mfge8-Cre containing BAC and original BAC were digested in the presence of XmaI (A)or SpeI (B), DNA fragments separated by electrophoresis and pictures taken at different time points (left:latest time point; right: earliest time point). (A) right side shows the loss of the XmaI fragment in the recombined clones (red arrow; 1.6kb), and the appearance of a second 3kb band (green arrow; stronger intensity band) which proves the proper insertion of the Cre GOI. Remaining restriction fragments (A, B) were identical to the original BAC and the predicted fragment size. * indicates undigested plasmid.

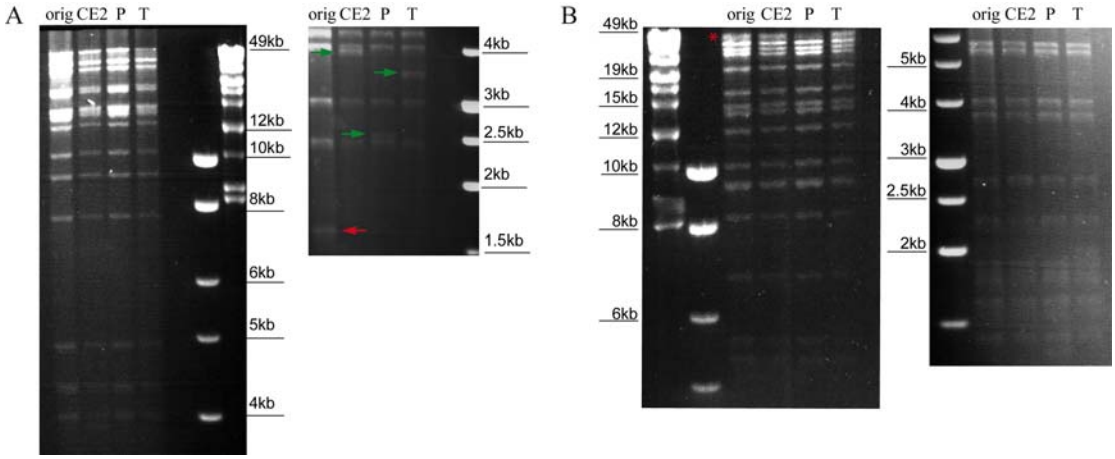


Fig. 45 Confirmation of Mfge8-CreERT2, -Prnp, -tdTomato integrity and modification by RFA
2ug of BAC constructs and original BAC were digested in the presence of XmaI (A)or SpeI (B), DNA fragments separated by electrophoresis and pictures taken at different time points (left:late time point; right: early time point). (A) right side shows the loss of the XmaI fragment in the recombined clones (red arrow; 1.6kb), and the appearance of the new fragment (green arrow; CreERT2 (CE2) 3.9kb, Prnp (P) 2.5kb, tdTomato (T) 3.4kb. Remaining restriction fragments (A, B) were identical to the original BAC and the predicted fragment. * indicates undigested BAC.

All constructs were shown to have maintained integrity and proper insertion by RFA. The specificity of the insertion and an area of about 9kb around the insertion of the GOIs Prnp, CreERT2 and tdTomato, were furthermore confirmed by Southern blot using radiolabelled probes annealing 5' and 3' of the insert (Fig. 46).

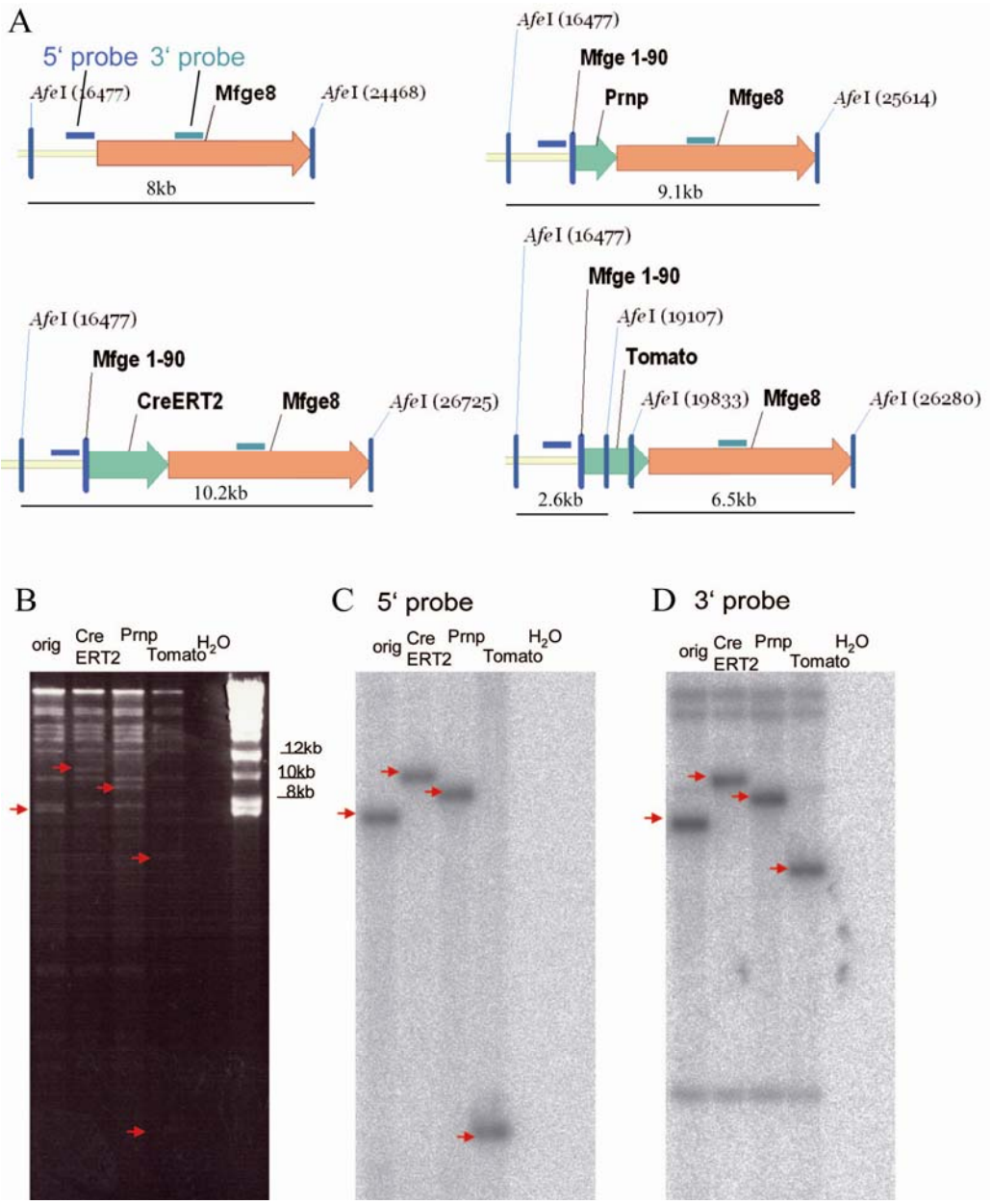


Fig. 46 Southern blot analysis of Mfge8-Prnp, Mfge-CreERT2, Mfge8-Tomato
(A) Scheme of the fragments that are generated by an enzymatic digest with AfeI of an unmodified BAC (left, upper row), a BAC with insertion of Prnp (right, upper row), CreERT2 (left, lower row) and tdTomato (lower row, right), the size of the fragments generated are indicated below the constructs, tdTomato contains 2 internal AfeI sites, resulting in the generation of 2 different fragments detected by the 5' and the 3' probe. (B) gel electrophoresis of the unmodified BAC (orig), Mfge8-CreERT2, Mfge8-Prnp, and Mfge8-tdTomato after digest with AfeI. Arrows point to the construct specific fragments generated by the digest. (C, D) Arrows point to the construct specific fragments that are detected with the radiolabelled 5' and 3' probe, respectively. 3' probe binds slightly unspecific, detecting additional fragments of the BAC.

DNA purification and pronuclear injection

DNA was linearized by a single cutting enzyme and run over a sepharose column. The principle of sepharose separation is similar to gel electrophoresis: smaller fragments pass through the column matrix quicker than large ones do. The sepharose purification is thus used to eliminate degraded and bacterial DNA from the full length BAC construct and equilibrate the DNA in injection buffer used during this procedure. Collected fractions were analyzed by pulsed field electrophoresis, which allows the separation of high molecular weight DNA (20-several hundred kb). The early fractions contained smaller bacterial DNA and sheared DNA, while full length DNA was found in later fractions (Fig. 47A, 48).

Mfge8-Cre fraction 8 (1ng/μL) was used for injection into male pronuclei of approx. 200 fertilized oocytes derived. Subsequent embryo transfer resulted in the birth of 21 live F0 pups. Tail biopsies of all F0 animals were taken and assessed for the presence of the transgene (Fig. 47 B). Two female F0 mice were identified as positive for the Mfge8-Cre transgene. Transgene-positive founder mice were bred to homozygous R26R-lacZ Cre reporter mice. However, neither one of the two founder mice managed to transmit the transgene to their offspring (21 mice were screened, Fig. 47 C and data not shown) suggesting that both founders were mosaic animals incapable of transgene germline transmission. The pronuclear injection with new DNA had therefore been repeated.

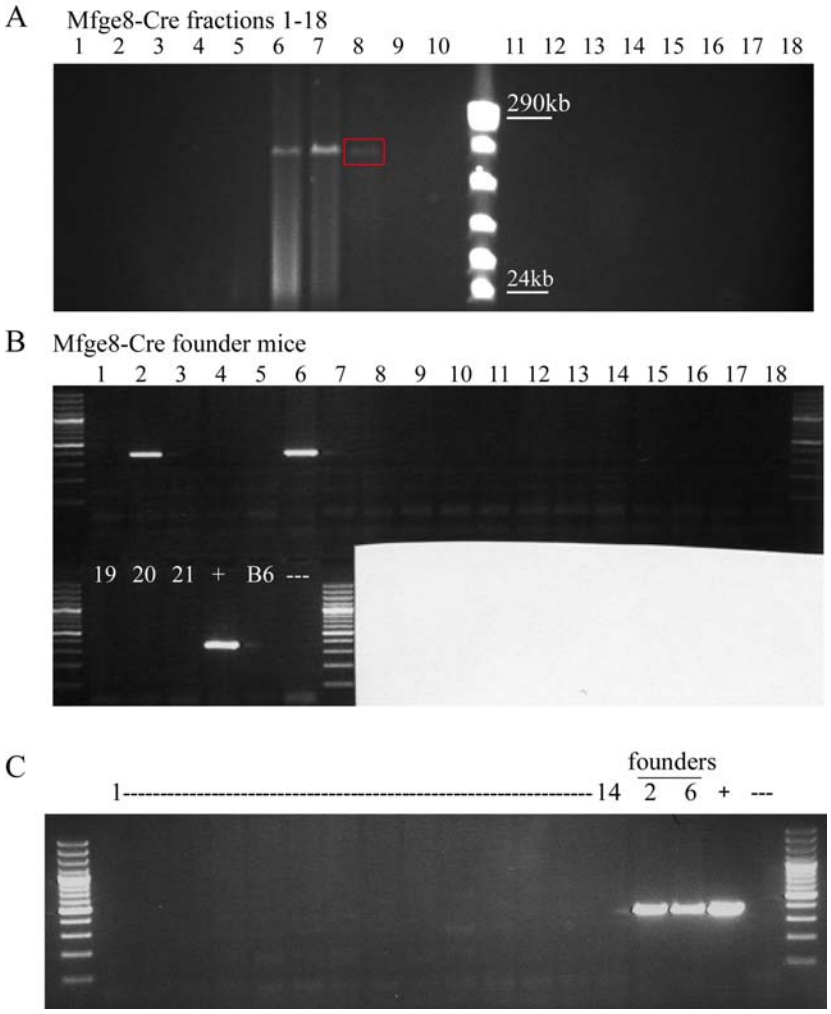


Fig. 47 Pulse field electrophoresis of Mfge8-Cre, analysis of founder mice
(A) Mfge8-Cre BAC was linearized with AscI, DNA purified over a sepharose column, and 22 fractions à 500uL were collected and fractions 1-18 were run on 1.1% agarose gel. Fractions 5-7 contained lower molecular weight DNA, while fraction 8 was enriched in full length plasmid. Fraction 8 (red box) was used for pronuclear injection. (B) Pups generated after pronuclear injection were analysed for the presence of the transgene using Cre specific primers. 2/21 mice were positive for the transgene (founder 2 and 6). (C) Offspring from the 2 founders (5, 9 mice respectively) were tested for transgene insertion by PCR. None of the offspring carried the transgene.

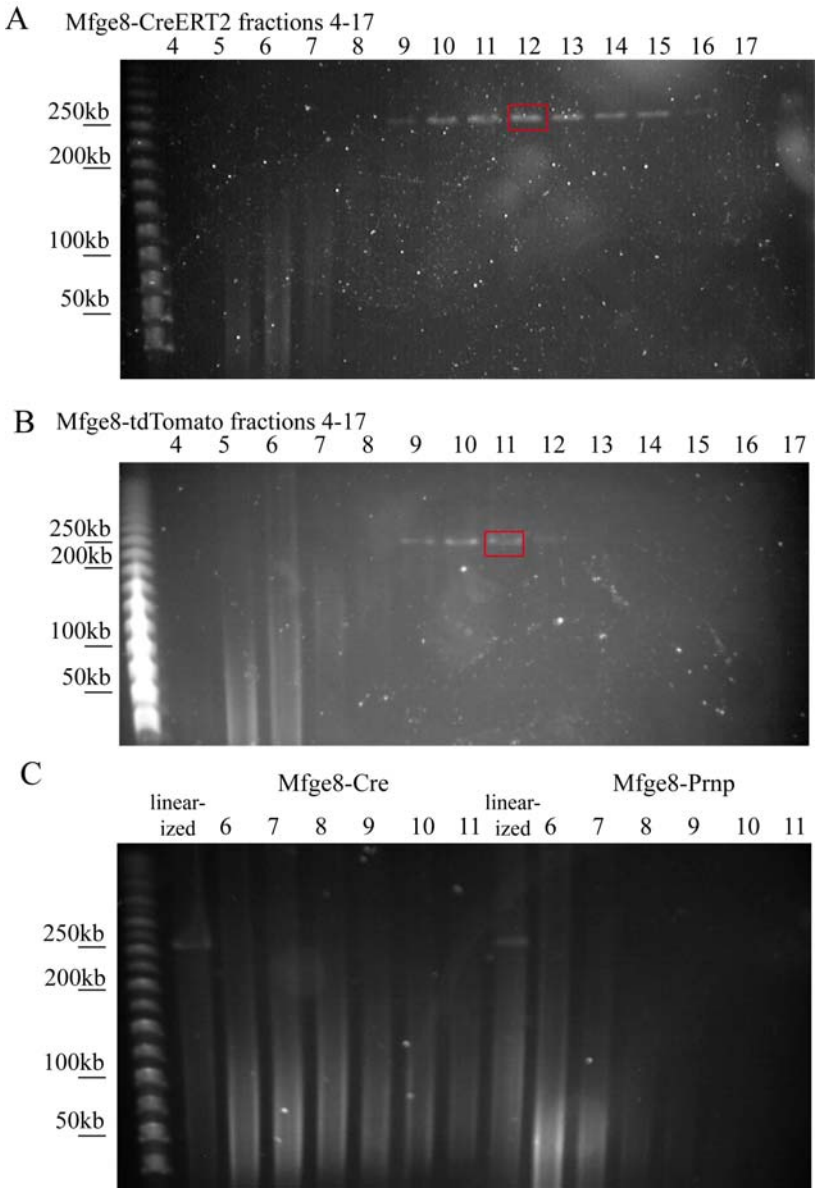


Fig. 48 Pulse field electrophoresis of Mfge8-CreERT2, Mfge8-tdTomato, Mfge8-Cre and Mfge8-Prnp

(A) Mfge8-CreERT2, Mfge8-tdTomato, Mfge8-Cre and Mfge8-Prnp BACs were linearized with MreI, DNA purified over a sepharose column, and 22 fractions à 500µL were collected. Fractions 4-17 of Mfge8-CreERT2 and Mfge8-tdTomato were run on 1.1% agarose gel (A, B). Fraction 12 of Mfge8-CreERT2 (1.9ng/uL) and fraction 11 of Mfge8-tdTomato (1.8ng/uL) contained mainly full length BAC and were used for pronuclear injection. (C) Mfge8-Cre and Mfge8-Prnp, linearized or linearized, sepharose purification fractions constructs (6-11). Sepharose purification resulted in degradation of the construct.

Fractions of linearized Mfge8-CreERT2, Mfge8-tdTomato, Mfge8-Cre and Mfge8-Prnp obtained after sepharose column separation were analyzed by pulse field electrophoresis (Fig. 39). Mfge8-CreERT2 faction 12 was used for pronuclear injection (1.9ng/uL), for Mfge8-tdTomato fraction 11 was used (1.8ng/uL). Due to DNA degradation during the sepharose separation step of Mfge8-Cre and Mfge8-Prnp, circular DNA was used for the injected into the pronucleus. 41 pups were received after injection of Mfge8-Cre into the pronucleus. PCR for Cre revealed that 3 out of 41 mice were transgene positive (Fig. 49).The pronuclear injection of Mfge8-Prnp yielded 50 pups, 7 were trangene positive (Fig. 50). We are currently waiting for the remaining pups derived from pronuclear injection of Mfge8-CreERT2, and Mfge8-tdTomato.

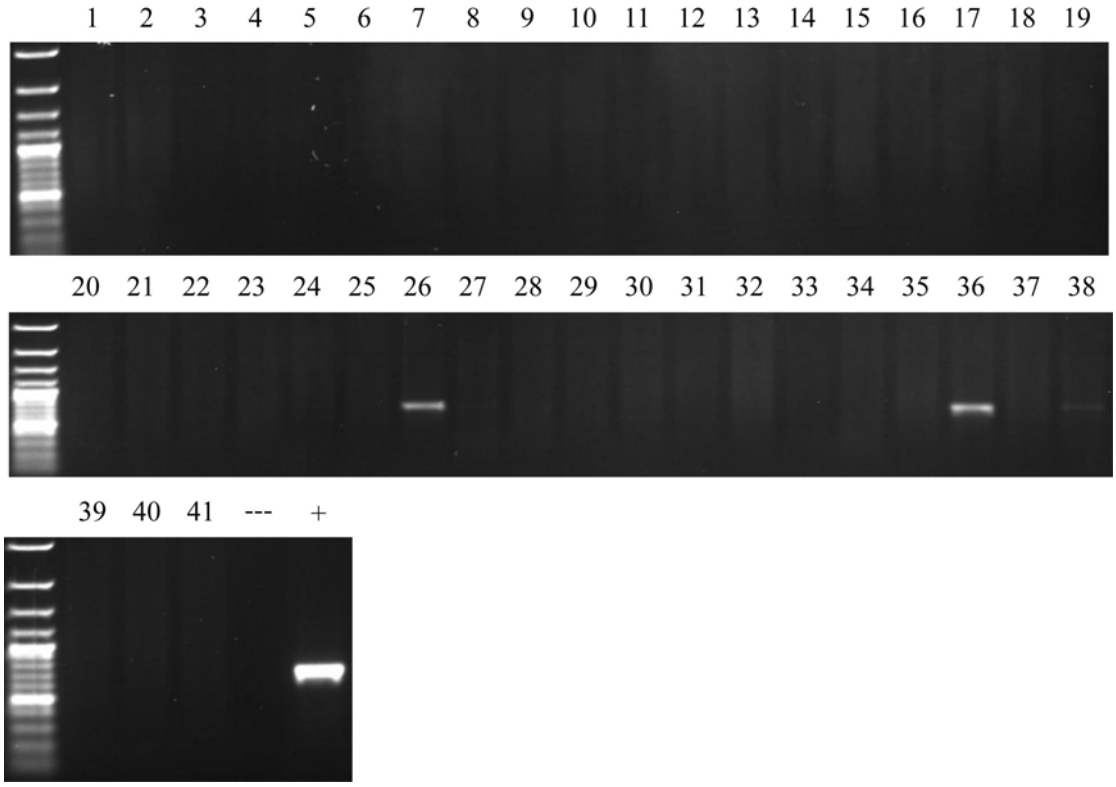


Fig. 49 Mfge8 Cre founders
Newborn mice derived from pronuclear injection of the Mfge8-Cre construct were analyzed for the integration of the transgene by PCR for Cre. 3/41 had insertion of the transgene (number 26, 36, 39). + positive control. --- water control.

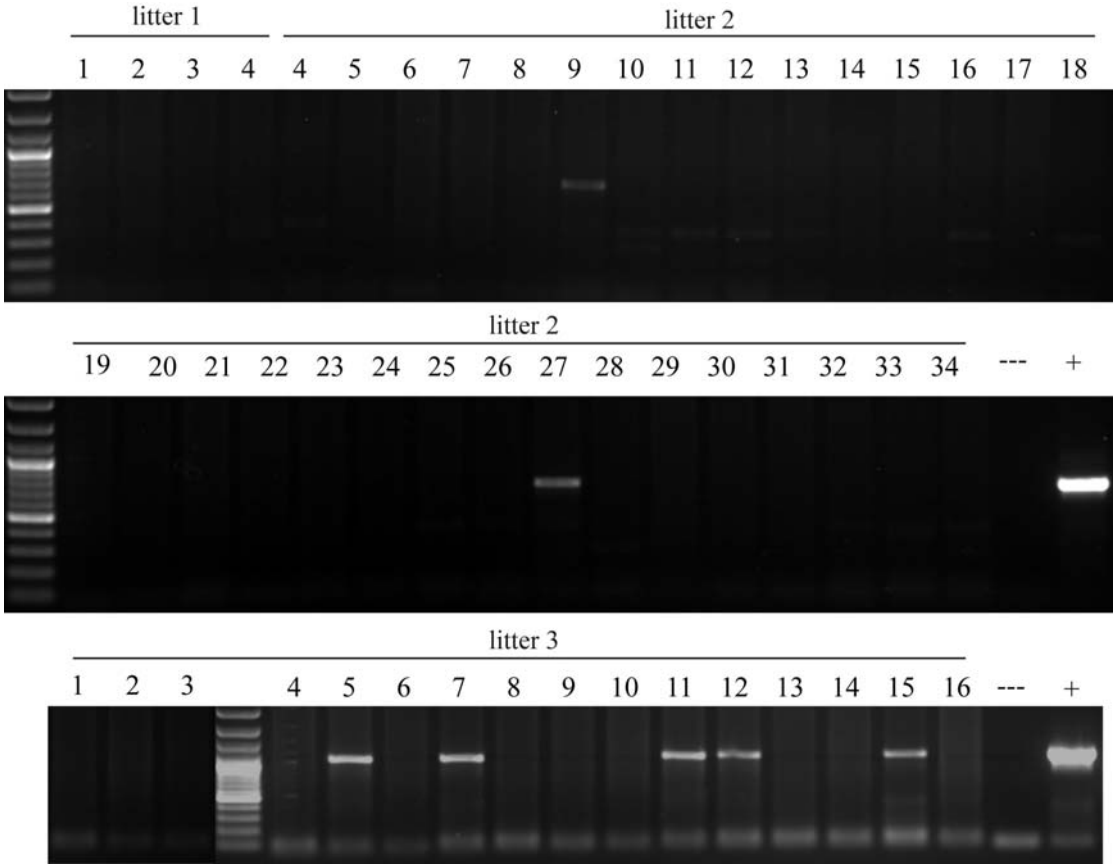


Fig. 50 Mfge8 Prnp founders
Newborn mice derived from pronuclear injection of the Mfge8-Prnp construct were assessed for the integration of the transgene by PCR for transgene specific Prnp. 7/50 had insertion of the transgene (litter2: 9, 27 litter 3: 5, 7, 11, 12, 15). + positive control. --- water control.

DISCUSSION

Mfge8 – a marker to study FDC-like cells

The origin of FDCs has remained issue of a long lasting debate. Using in situ hybridization techniques for *Mfge8*, the RNA encoding for the FDC marker FDC-M1, I found that apart from mature FDCs, additional cells express this marker. They were localized within the B cell follicle, lining the marginal sinus, but were also to some minor extent within the T cell zone of WT spleens. Similar results were obtained from the analysis of WT LNs, where cells outside the B cell follicle expressed Mfge8. More detailed analysis of the spleens revealed that these cells shared additional markers with mature FDCs, such as BLC, ICAM-1, MAdCAM, and BP-3.While Mfge8 stained only very specific cell populations in the MS, BP-3 and ICAM-1 showed a much broader expression pattern in the T cell zone and in case of ICAM-1 also in the red pulp. I concluded from that that the *Mfge8*$^{+}$ cells in the MS, though expressing follicular stromal cell markers, could represent a specific population, with characteristics of FDCs. These cells *Mfge8*$^{+}$ cells were radioresistant, their maintenance depended on LTβR signaling and they were derived from the stromal compartment after BM reconstitution experiments, supporting furthermore their similarity to mature FDCs. The progenitor of FDC so far had remained unknown, we therefore assumed these cells putative precursors and called them preFDCs. As the preFDCs were also producing factors such as BLC and ICAM-1, there was a high probability that they were also involved in the regulation of B cell attraction. Indeed we found that the dedifferentiation of preFDCs, by blocking the LTβR signaling cascade, went hand in hand with a loss of MZ B cells. To finally prove that preFDCs are cells needed to establish the MZ B cell zone, conditional ablation of the preFDC population would be essential. This will be pursued using the Mfge8-Cre transgenic mice, crossed to the Cre-inducible diphtheria toxin receptor mice (iDTR). Why preFDCs produce Mfge8 remains an open question. In contrast to GCs, apoptosis in the MZ is not a frequent event. Other functions were so far attributed to Mfge8 -

Mfge8 is involved in cell-cell adhesion, e.g. in the epdidymis Mfge8 regulates the intercellular adhesion of epithelial cells in an integrin αv dependent fashion. Mfge8 was also shown to be present in exosomes, and could therefore regulate transfer of membranous particles, for example containing ICs, and could probably modulate the immune response. Another described function of Mfge8 is the facilitation of neovascularization, and Mfge8 is also present in fully developed vessels. Since the MS is part of the splenic vasculature, preFDCs secreting Mfge8 might regulate the local vascular function. So far no MZ defect was described in *Mfge8*$^{-/-}$ mice. This might be either due to overlapping functions with other molecules as it has been described for the adhesion molecules ICAM-1 and VCAM-1. Only the blockage of both will result in the loss of the MZ B cells. It might be interesting to analyze the migration of hematopoietic cells and the transfer of ICs in *Mfge8*$^{-/-}$ mice. The finding of cells with FDC characteristics further implied that we are dealing with the long searched precursors of FDCs.

Cellular and molecular cues needed for putative precursors of FDCs

In WT spleens preFDCs and mature FDCs were co-existent, and I wanted to determine the molecular and cellular cues they depended on. I therefore tested the presence of preFDCs in genetically modified mice, which lack mature FDCs. Surprisingly, the ablation of TNFR1 did not alter the presence of preFDCs, and mice lacking B cells (Rag1$^{-/-}$ and μMT^{D}) contained preFDCs, as well, indicating that only mature FDCs depend on these lymphocytes and signaling mechanisms. The majority of preFDCs, however, depended on LTβR signaling or on B cells in combination with the common cytokine receptor gamma (Rag2$^{-/-}$γc$^{-/-}$), though in their absence some cells expressing *Blc* and *Mfge8* remained, which I termed proFDC. Interestingly, similar observations were made in LN development: even though the maturation of LNs needs the presence of LTs and B cells, the stromal organizer secreting BLC at the future LN site is independent of LTβR activation (Eberl et al. 2004; van de Pavert et al. 2009). Upon influx of LTis, LTβR signaling is induced, leading to the expansion of stromal organizer cell population, upregulation of chemokines, adhesion molecules, and lymphangiogenesis.

That the defect in the generation of preFDCs seen in Rag2$^{-/-}$γc$^{-/-}$ was due to a deficiency in LTs was supported by the finding that spleens of these mice had reduced

Lta mRNA and that agonistic LTβR stimulation using anti-LTβR antibodies would lead to an increase in FDC-like cells present. I therefore conclude that the early proFDC is independent of LTβR activation, B cells and the common cytokine receptor gamma chain (γc), but expansion of proFDCs depends on them. In the absence of the γc chain the signaling of different IL-Rs is abrogated and NK cells not generated. However, though NK cells were reported to produce LTs under certain conditions, I was surprised to see that the ablation of NK cells from $Rag1^{-/-}$ mice had no effect on the development of preFDCs. IL-7R signaling is also blocked in the absence of the γc. LTis, which express the IL-7R, are reduced in number and their function impaired in $IL\text{-}7R^{-/-}$ and $Rag2^{-/-}\gamma c^{-/-}$ mice. I found that $Rag1^{-/-}$ mice depleted from LTis, had significantly reduced numbers of preFDCs. I therefore conclude that LTis supply preFDCs with LTs needed for the induction and for the development from the proFDC stage. The splenic generation of proFDCs, preFDCs and FDCs is therefore highly similar to LN maturation – I observed that the same signaling and cellular mechanisms direct the discrete steps of development.

Perivascular induction of FDCs

In mice lacking all follicular structures ($Rag2^{-/-}\gamma c^{-/-}$), proFDCs cluster at perivascular sites in the spleen. Reconstitution with WT BM of $Rag2^{-/-}\gamma c^{-/-}$ mice leads to the restoration of all hematopoietic cells and sources of LT and results in the development of B cell follicles. In this experimental setup I found that lymphocyte entry happened at sites where proFDCs were present and that the infiltration of blood cells resulted increased proFDC numbers at perivascular sites. Furthermore, concomitantly with the generation of follicular structures, $Mfge8^{+}$ cells were lining the future MS area and present within the follicles, indicating that proFDCs including future FDCs originate from the same perivascular cell. The process of proFDC expansion upon reconstitution depended on LTβR activation, most probably with B cells providing the LT ligands, as shown by BM reconstitution experiments with cells from $Lta^{-/-}$ mice. The study of neonatal spleen development confirmed that the perivascular expansion of $Mfge8^{+}$ proFDCs happened simultaneously with the entry of lymphocytes at early postnatal stages. Unpublished results and previous studies suggest that FDCs are derived from a mesenchymal local cell. Our results furthermore indicated that they originate from a perivascular local cell. Perivascular $Mfge8^{+}Blc^{+}$ cells might be

important to control the local entry of the lymphocytes by chemokines like BLC. In addition we observed that during the white pulp development, *Mfge8* RNA was restricted to perivascular sites, while Mfge8 protein was mainly found within the vascular structures (data not shown). This indicates that Mfge8 might have a role in the lymphangiogenesis. A role of Mfge8 in neovascularization was already described previously, where Mfge8 was reported to bind to integrin αv/β3 and αv/β5 of endothelial cells, resulting in phosphorylation of AKT and Erk and activation of the pro-angiogenic pathway (Silvestre et al. 2005).

Pericyte origin and pericyte character of preFDCs

Pericytes are perivascular cells, which ensheath the endothelial cells. They regulate vascular development, stabilization and maturation, lack of pericytes is perinatally lethal (Soriano 1994; Armulik et al. 2005). Pericytes, were furthermore shown to be rather undifferentiated cells, which can undergo adipogenesis, chondrogenesis, osteogenesis, and myogenesis (Dellavalle et al. 2007; Tang et al. 2008). Pericyte markers are SMA, NG2, and PDGFRβ. I therefore assessed whether FDCs and preFDCs express any pericyte markers. Mature FDCs expressed no or little pericyte markers, however, co-localization of Mfge8 expressing preFDCs with the pericyte markers SMA and PDGFRβ was found. This indicates that preFDCs are still more pericyte like, and that they might reflect a transient form between the pericyte and the mature FDC. Though mature FDCs did not express any pericyte markers, we wanted to test whether FDCs still might have derived from pericyte cells. I used the pericyte reporter mouse line, Pdgfrb-Cre crossed to the Cre-reporter line Rosa26R lacZ and analyzed the expression in the spleen. I found that the expression of the reporter co-localized with FDC markers. Thus FDCs must have expressed the pericyte marker during their development, which supports their perivascular, pericyte origin.

Mfge8 expressing cells in organs where FDCs can be generated ectopically

FDCs can be generated ectopically in non-lymphoid organs during chronic inflammatory conditions. In mouse models ectopic FDCs emerge as a consequence of transgenic overexpression of in liver and kidney. We therefore tested whether Mfge8

expressing cells could be found in kidney and liver of WT mice. In the kidney, *Mfge8*$^{+}$ cells were found in the glomeruli and lining the tubuli of the kidney. Interestingly, mesangial cells are located within the glomeruli. They are specialized pericyte cells, which also have phagocytic activity and were shown to ingest apoptotic cells (Cortes-Hernandez et al. 2002) They were furthermore shown to phagocytose immuneglobulins via their Fc-receptors (Sedor et al. 1987; Santiago et al. 1989; Santiago et al. 1991). Thus Mfge8 expressing cells in the kidney might be the glomerular pericytes, and Mfge8 might regulate the removal of proteins and apoptotic cells in this context. That further chronic activation of the LTβR results in the generation of mature FDCs in this organ might imply that the local mesangial cells undergo differentiation. This question will be tackled using the Mfge-CreERT2 mouse line in combination with a R26R reporter mouse, to conditionally and permanently label mesangial cells and follow their fate during the development of kidney TLOs.

In the liver of WT mice, I found expression of Mfge8 by oval cells, which happened in an LTβR activation independent manner. Upon stimulation with agonistically acting anti-LTβR antibodies, Mfge8 was found to be upregulated in oval cells, but also in a stellate pattern throughout the liver tissue, which might reflect another cell type, since there was no overlay with the oval cell marker to be found. Stellate cells are liver pericytes, and could be the cells upregulating Mfge8 upon LTβR activation. I will examine the co-localization of Mfge8 with the pericyte and stellate cell marker SMA in agonist treated livers. Interestingly, LTβR activation in non-matured lymphoid organs such as the Rag2$^{-/-}$γc$^{-/-}$ and in the liver, a non-lymphoid organ, results in the upregulation and appearance of Mfge8 expressing cells. These results support the model of a common FDC precursor in lymphoid and non-lymphoid tissues, whose development and maturation depends on LT signaling.

Though lymphocytes can infiltrate many organs during chronic inflammations, not all organs do develop structured follicles with FDCs present. Are differences in the pericyte pool of a specific organ responsible for the outcome of TLO development? In case of adipogenesis it could be shown that only pericytes isolated from the adipocyte tissue contained precursor potential for white fat cells. To study different non-lymphoid organs with/without LTβR activation for the presence of Mfge8^{+} cells might give insights into the ectopic development of TLOs.

Concluding remarks, an FDC maturation model and outlook

While the signaling mechanism and the cells involved in the maturation of SLOs and TLOs are established, little is known about components tipping the scales for their very first induction. A recent publication suggests that neuronal endings give instructive signals to organizer cells at sites of future LNs, which, in turn, produce the B cell attracting chemokine BLC. BLC leads to the first recruitment of lymphoid tissue inducer cells, which results in upregulation of chemokines and lymphangiogenic factors and in the influx of lymphocytes (van de Pavert et al. 2009). In TLOs the first induction seems to be mediated by the antigen stimulating innate and adaptive immune responses which manifest in the influx of lymphocytes, the generation of follicular structures and the differentiation of the vasculature into HEVs regulating the influx of lymphocytes (Ludewig et al. 1998; Cupedo et al. 2004; Aloisi and Pujol-Borrell 2006).

I suggest that at future sites of SLOs and TLOs, pericytes get an instructive signal, be it the presence retinoic acid released close-by neuronal endings in case of LNs (and probably also in the spleen) or a chronic exposure to an antigen stimulating the innate immune cells. Activation of these pericytes in turn leads to their upregulation of Mfge8 and BLC - in LNs they are now called stromal organizers, in the spleen I would like to introduce the term progenitor FDCs (proFDCs) - which leads to the recruitment of LTi cells and lymphocytes (Fig. 51, Fig. 52). Pericytes furthermore communicate with the vascular endothelial cells, upregulate adhesion molecules and lymphangiogenic factors. As a consequence of the positive feedback loop even more hematopoietic cells expressing high amounts of LTs arrive at the site of the developing lymphoid organ, activation of the LTβR induces an expansion of $Mfge8^{+}Blc^{+}$ proFDC population, some of them might regulate the further angiogenesis, while others differentiate into precursors FDCs (preFDCs) in the marginal sinus and within the white pulp and mature FDCs. The progression from the proFDC stage to the preFDCs needs functional LTis and the presence of LTβR ligands, but B, T and NK cells and TNFR1 activation are not essential. Presumably, the low levels of LTs and other factors produced by LTis suffice for the initial differentiation of proFDCs to preFDCs. Maturation of FDCs, is given only in the presence of B cells, which are providing high amounts of LTs and TNFR1 signaling.

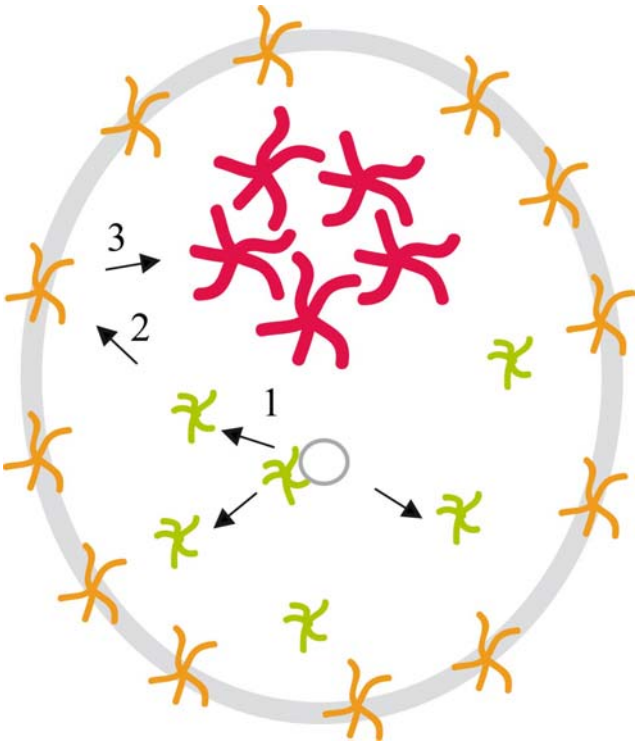


Fig. 51 Scheme of FDCs development in the splenic follicle according to Mfge8 expression
Perivascular cells (proFDCs, green) lining the central arteriole of the future splenic follicle. Upon influx of LTis, the increase in lymphotoxin present and generation of lymphoid follicles, perivascular proFDCs expand, are present within the follicular structure (1) as well as in the marginal sinus (2, preFDCs, orange). In the presence of TNFα and B cells further differentiate into mature FDCs of the primary and secondary lymphoid follicles (3, red)

I have no final proof for the succession line of proFDCs - preFDCs - mature FDC, though many observations support this hypothesis. To verify that proFDCs indeed differentiate into mature FDCs, I have started to generate BAC transgenic mice, which can be used to lineage trace FDCs. Mfge8-CreERT2 mice crossed to the R26R mice will be used to pulse Mfge8 expressing perivascular proFDCs by short term tamoxifen treatment at early postnatal stages, mature FDCs will be assessed for the expression of the reporter.

Furthermore, PDGFRβ-Cre mice are currently bred to mice with a loxP flanked Ltbr locus. This will result in the conditional ablation of LTβR in PDGFRβ expressing cells. FDC development requires LTβR activation. If FDCs are indeed derived from PDGFRβ expressing cells, they will not obtain essential LTβR activation and will be blocked in their development.

I want to highlight that I do not believe that the only function of the pro- and preFDCs is the generation of mature FDCs, the use of the LTβR decoy receptor to compete for endogenous LTβR signaling, showed that MS preFDCs might function in the regulation of MZ B cell recruitment in the mature spleen. Therefore, preFDCs could regulate in an indirect way the TI-antigen response in the MS by cognate MZ B cells, but also the transfer of antigen by non-cognate MZ B cells into the germinal centers.

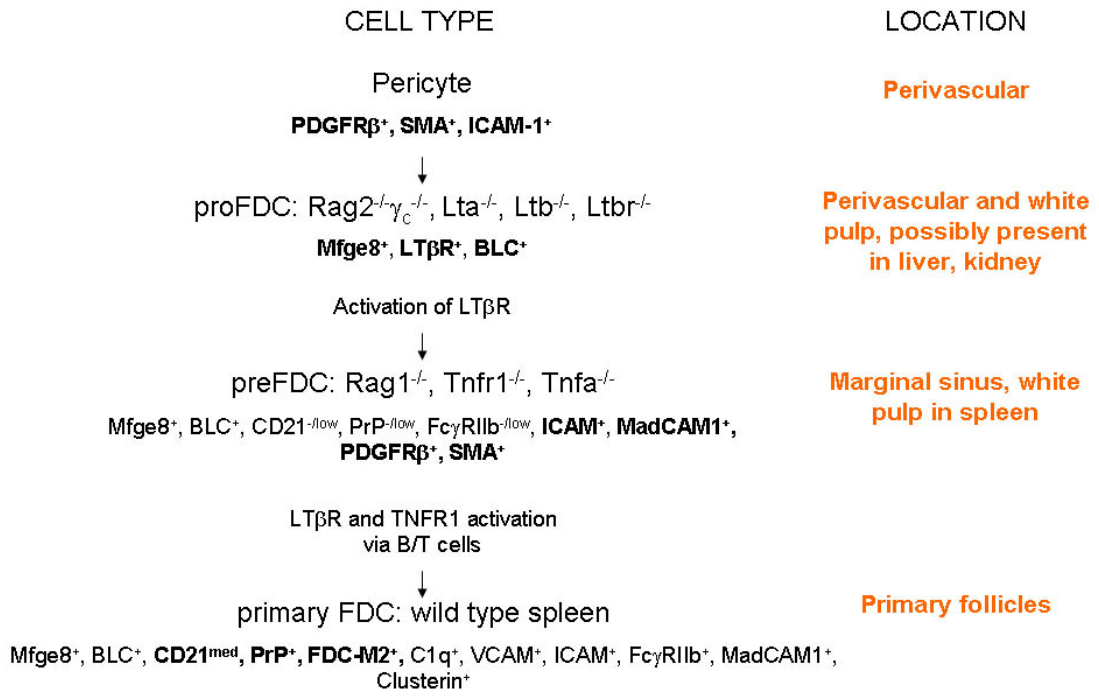


Fig. 52 Flow chart summarizing the FDC development stages
Cell type, cell type subtypes on the left, location is indicated on the right. Low differentiated pericyte is at the beginning of the chart, the mature primary FDC at the end. Specific/novel cells markers, which define a certain development stage are indicated in bold and with a +. -/low: under detection limit

Materials and Methods

Mice

C57BL/6 were obtained from Harlan and Rorc(γt)$^{GFP/GFP}$ from Jackson Laboratories (Eberl and Littman 2003). Rag1$^{-/-}$, Rag2$^{-/-}$γc$^{-/-}$, Tnfr1$^{-/-}$, Lta$^{-/-}$, Ltb$^{-/-}$, Ltbr$^{-/-}$, Mfge8$^{-/-}$ and C57BL/6 × 129Sv were maintained under specific pathogen free conditions (Mombaerts et al. 1992; De Togni et al. 1994; Alimzhanov et al. 1997; Futterer et al. 1998; Goldman et al. 1998; Hanayama et al. 2004). Rag1$^{-/-}$ were intercrossed with Rorc(γt)$^{GFP/GFP}$ to receive Rag1$^{-/-}$Rorc(γt)$^{GFP/GFP}$ and Rag1$^{-/-}$Rorc(γt)$^{GFP/+}$. Spleens from Tnfa$^{-/-}$ and μMT^{D} were obtained from Rolf Zinkernagel's laboratory (Kitamura et al. 1991; Rothe et al. 1993). Pdgfrb-Cre^{+}R26RlacZ^{+} and Pdgfrb-Cre^{-}R26RlacZ^{+} spleens were obtained from Michelle Tallquist. All experiments were in accordance with Swiss federal legislation and had been approved by the local authorities.

Generation of BM chimeras and immunizations

BM recipients were lethally irradiated (950 rad). Donor BM was isolated by flushing tibias and femurs. Recipient mice received 107 donor BM cells intravenously (i.v.). Reconstitution efficiency was assed after 5 weeks by FACS analysis of blood leukocytes. 6 weeks after engraftment, mice were immunized intraperitoneally (i.p.) with 100 μg OVA (Sigma-Aldrich) in alum (Imject Alum; Thermo Fisher Scientific). 2 weeks later, mice were boosted with the same dose of OVA. For reconstitution of 6-8 week old Rag2$^{-/-}$γc$^{-/-}$ mice received 2x10^{7} donor bone marrow cells without previous irradiation, mice were sacrificed and organs taken at indicated time points.

Antibody and LTβR-Ig treatment

For all treatments 8-12 week old mice were used.

To block LTβR signaling mice were either treated i.v. 2x with 50μg with m LTβR - mIgG1 (Biogen) or isotype control (MOPC 21, Biogen) during a period of a week or i.p. with 100ug once a week over a period of 4 weeks. For agonistic LTβR treatment 50ug of anti- LTβR antibody (AC.H6, Biogen) or hamster IgG (Ha4/8-3.1, Biogen) were injected i.v. and mice sacrificed after 24h. Depletion of NK cells was performed using 100μg anti-mouse NK1.1 (PK126, BD biosciences) or mouse IgG2a (G155-178, BD biosciences) as isotype control injected i.v. weekly over a period of 3 weeks.

For depletion of MZ B cells WT mice were treated i.p. with 100μg anti-integrin αL in PBS (clone M17/4; eBioscience, rat IgG2aκ) in combination with either 100μg anti-VCAM1 (clone M/K-2; SouthernBiotech; rat IgG1κ) or 100μg anti-integrin α4 (clone PS/2; SouthernBiotech, rat IgG2bκ). Isotype controls received 100ug rat IgG2aκ (eBioscience) in combination with either 100ug rat IgG1κ or 100ug rat IgG2bκ (both SouthernBiotech). Mice were sacrificed after 4 days.

Immunohistochemical analysis

Cryosections were stained with hematoxylin/eosin or with antibodies against CD3 (500A2), B220 (RA3-6B2) and CD21/35 (7G6, all BD bioscience) and detection was performed using respective AP- coupled secondary and tertiary antibodies in combination with Fast Red staining kit (Sigma). For immunofluorescent stainins splenic cryosections were acetone fixed, blocked in phosphate buffered saline (PBS) containing 0.5% BSA and 1% goat serum and stained with primary antibodies added: anti-Mfge8 (2422, Alexis Corporation or 18A2, MBL), anti-ICAM1-PE (3E2, BD bioscience), anti-SMA-PE (Sigma), anti-NG2, anti-MadCAM (MECA-367, BD bioscience), anti-MOMA1-FITC (Serotec), anti-ERTR9 (Bachem), anti-CD16/32 (2.4G2, BD bioscience), anti-PrP (XN, made in our laboratory (Montrasio et al. 2000)), anti-CD21/35 FITC (7G6, BD bioscience), anti-PDGFRβ (ebioscience) and the secondary antibodies anti-rabbit Alexa546, anti-rat Alexa594, anti-rat Alexa 647, anti-hamster Alexa 546, anti-hamster Alexa647, streptavidin Alexa 647 (all Invitrogen), counterstained with Dapi and analyzed by fluorescence microscopy (BX61, Olympus).

In situ RNA Hybridization

For generation of the RNA probe against Mfge8 and Blc, the open reading frame of Mfge8 and Blc was cloned into pBluescript II KS+ (Stratagene). After plasmid linearization RNA transcription was performed according to manufacture's instructions (DIG RNA Labeling Kit, Roche) and the DIG (digoxigenin)-labeled RNA was hydrolyzed in carbonate buffer (40 mM $NaHCO_3$, 60 mM Na_2CO_3). Frozen spleen sections (7 μm) were thaw-mounted, air-dried and post-fixed in 4% paraformaldehyde in PBS followed by acetylation with 0.5% acetic anhydride in 0.1 M triethanolamine. Hybridization was carried out in buffer containing 50%

formamide, 5x Saline Sodium Citrate (SSC) solution, 100 μg/ml yeast t-RNA (Roche) and 5x Denhardt's solution. For in situ RNA hybridization 200 ng/ml DIG-labeled RNA probe was added to the hybridization buffer and incubated at 72°C overnight. For detection either anti-DIG-AP antibodies were used and staining using BCIP/NBT.

β-Galactosidase Staining

Frozen cryosections were air dried, fixed in 0.4% glutaraldehyde PBS, and permeabilized with 0.02% NP-40, 0.01% sodiumdeoxycholate, 2mM $MgCl_2$ in PBS. Sections were incubated for 48h at 37°C shaking in the dark in staining solution (5mM FeKCN(II), 5mM FeKCN(III), 2mM $MgCl_2$ in PBS containing 1mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)).

FACS

Single cell suspensions were made from freshly isolated spleens in PBS buffer containing 2% fetal calf serum (FCS), red blood cells lysis was performed in RBC lysis buffer (eBioscience). Fc-receptors were blocked with anti-CD16/32 antibody (2.4G2) and subsequently stained with following antibodies: NK1.1-APC (clone PK136), CD49b-PE (DX5), CD21/35-FITC (7G6), CD23-PE (B3B4). CD19-Alexa647 (selfmade, Clone ID3), CD3-PE (500A2), CD4-Percp (RM4-5), biotinylated IL-7R (A7R34, eBioscience) and streptavidin-Alexa647 (Invitrogen). All antibodies were obtained from BD biosciences unless indicated. Cells were analyzed by FACSCalibur flow cytometer and FlowJo software.

Quantitative PCR

Total RNA from spleens was isolated using Trizol (Invitrogen) and chloroform extraction. 1μg RNA was used to generate cDNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instruction. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Qiagen AG, Switzerland) on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using default cycling conditions. Expression levels were normalized using Gapdh.

Gapdh forward primer: 5'-CCACCCCAGCAAGGAGACT-3'
Gapdh reverse primer: 5'-GAAATTGTGAGGGAGATGCT-3'

Mfge8 forward primer: 5'-ATATGGGTTTCATGGGCTTG-3'
Mfge8 reverse primer: 5'-GAGGCTGTAAGCCACCTTGA-3'
Cd21 forward primer: 5'-CCCATAGTA CCAGGGGGATT-3'
Cd21 reverse primer: 5'-AGACTGGCAGAGCTGTGGGT-3'
Clusterin forward primer: 5'-CCAGTTCCCAGACGTTGATT-3'
Clusterin reverse primer: 5'-AGCAGGGATGAGGTGTGGAG-3'
Blc forward primer: 5'-TCGTGCCAAATGGTTACAAA -3'
Blc reverse primer: 5'- ACAAGGATGTGGGTTGGGTA-3'
Ifgbp3 forward primer: 5'-CGAGTCTAAGCGGGAGACAG-3'
Ifgbp3 reverse primer: 5'-TTGTTGGCAGTCTTTTGTGC-3'
Cd30l forward primer: 5'-TGGCGATCATTCTGGTACTG-3'
Cd30l reverse primer: 5'-5'CAAGCCAGGGAATTGGACTA-3'
Lta forward primer: 5'-TCCACTCCCTCAGAAGCACT-3'
Lta reverse primer: 5'-AGAGAAGCCATGTCGGAGAA-3'
Tnfa forward primer: 5'-CGATGGGTTGTACCTTGTC-3'
Tnfa reverse primer: 5'-CGGACTCCGCAAAGTCTAAG-3'

BAC BAC RP23-24N16

E coli containing the BAC RP23-24N16 were obtained from BACPAC Resources Center. It contained a 250kb insert of female mouse genomic DNA (C57BL/6J background). Vector insert was generated by partial digest using EcoRI and EcoRI methylase and ligated into the pBACe3.6 vector containing a ChloR. BAC clone had been fully sequenced and sequence published (NCBI).

Generation of plasmids for the targeting cassette

Plasmids containing Cre recombinase or CreERT2 followed by polyA sequence and FRT flanked neomycine resistance (NeoR) gene were obtained from Thorsten Buch. Subcloning of Prnp, tdTomato, Lta and Ltb open reading frame was done by addition of restriction motifs by PCR and ligation into Cre containing plasmid using Rapid DNA Ligation Kit (Roche) and BstbI, MluI, KpnI enzymes (NEB).

Prnp forward primer: 5'**gctctt-cgaa**accatggcgaaccttggcta 3' (bold BstbI)
Prnp reverse primer: 5'**gctca-cgcgt**tcatcccacgatcaggaaga 3' (bold MluI)

Lta forward primer: 5'**gctctt-cgaa**accatgacactgctcggccg 3**'** (bold BstbI)
Lta reverse primer: 5'**gctca-cgcgt**ctacagtgcaaaggctccaa 3'(bold MluI)
Ltb forward primer: 5'**gctcggtac-ca**ccatggggacacggggactgcagg 3' (bold KpnI)
Ltb reverse primer: 5'**gctca-cgcgt** tcaccccaccatcaccgc 3'(bold MluI)
tdTomato forward primer: 5' **gctcggtac-ca**ccatggtgagcaagggcga 3'(bold KpnI)
tdTomato reverse primer: 5'**gctca-cgcgt**ttacttgtacagctcgtcca 3'(bold MluI)

Generation of the targeting cassettes

Addition of homology arms was done using primers with approx. 25bp homology to the plasmid (Cre, CreERT2, tdTomato, Prnp, Ltb) and 50bp identical sequence of the Mfge8 Exon1 targeting area. For PCR amplification high fidelity DNA polymerase Phusion (Finnzymes) was used with Expand Long Template Buffer 3 (Roche), dNTPs (200μM) and 0.5μM primer in presence of 5ng plasmid. Optimal amplification was determined by gradient PCR thermocycler.

Primers used to add homology arms: bold areas indicate homology area with Mfge8 Exon 1 and Mfge8 Intron 1 in the forward, reverse primer, respectively

Primer Mfge8–Cre forward
5'**tctcgagtcccagcatcagagcgcgtggacctttcccgcgtcccgc**accatgcccaagaagaagaggaaggtgt 3'
Primer Mfge8–Ltb forward
5'**actctcgagtcccagcatcagagcgcgtggacctttcccgcgtcccgc**accatggggacacggggactgcag 3'
Primer Mfge8–CreERT2 forward
5'**tctcgagtcccagcatcagagcgcgtggacctttcccgcgtcccgc**accatgggcgccacgagtgatgaggttc 3'
Primer Mfge8–Lta forward
5'**tctcgagtcccagcatcagagcgcgtggacctttcccgcgtcccgc**accatgacactgctcggccg 3'
Primer Mfge8–tdTomato forward
5'**tctcgagtcccagcatcagagcgcgtggacctttcccgcgtcccgc**accatggtgagcaagggcgaggaggtca 3'
Primer Mfge8–insert reverse (for all constructs the same)
5'**gagaacttccgagcggcgggcagaacaggggcgaggccagagggactcac**cttaagcttggaaaagctgg 3'

PCR products were analyzed on a 1%agarose gel containing ethidiumbromide, gel extracted using GFX PCR DNA and gel extraction kit (GE healthcare).

Homologous recombination

E.coli containing BAC RP23-24N16 were electroporated (1350V, 10uF, 600Ohms Eppendorf Electroporator 2510, 1mm cuvette) with Red/ET plasmid containing tetracycline resistance gene, a temperature sensitive origin of replication and a L-arabinose dependent expression of Red recombinases (pSC101-BAD-gbaA genebridges). Clones were selected by growth on LB agar plates containing 3µg/mL tetracycline (Sigma) and 15µg/mL Chlor (Sigma). Single clones were then grown in 1.4mL LB broth in the presence of Chlor only at 30°C until they reached an optical density at 600nm (OD_{600}) of 0.2-0.3, then 50µL 10% L-arabinose (Sigma) was added, temperature increased to at 37°C for 40min until to induce transcription of recombinase proteins, 200ng of targeting cassette containing NeoR and the homology arms were electroporated and selected on LB agar plates containing Chlor (15µg/mL) and Kana (15µg/mL; Sigma) and grown at 37°C to loose Red/ET plasmid. The loss of Red/ET plasmid was confirmed by the inability of these clones to persist in the presence of tetracycline containing LB broth. Recombination of cassettes into the Mfge8 Exon1 locus was confirmed by PCR and sequencing.

Removal of NeoR by Flp recombinase

Single recombined clones were grown 3h at 37°C in LB broth containing Chlor and Kana. Then electroporated with Flp plasmid (Flp706-tet genebridges), which contains a TetR gene, a thermosensitive origin of replication (lost at 37°C), as well as a heat inducible transcription of the Flp (induced at 37°C). E. coli were selected on LB agar containing tetracycline, Chlor and Kana. Single clones were picked and grown over night in 1mL LB broth (containing Chlor) at 37°C, which is leading to Flp mediated excision of NeoR gene, concomitant with a loss of the Flp-plasmid. 60-80% of the clones should have undergone recombination, which is assessed by growing same amounts of E.coli on LB agar plates containing, both, Chlor as well as Kana, or Chlor only and comparison of the total numbers in colonies grown. Due to recombination of NeoR, more E.coli were present on Chlor LB agar plates. 10 clones per construct were picked from Chlor containing agar plate, expanded in LB broth and tested for the loss of NeoR by PCR as well as by growing clonal replicas in LB broth containing Kana.

Restriction fragment analysis (RFA)

2-3µg of BAC DNA were digested in presence of XmaI, SpeI or BssHII (Roche Applied Bioscience) according to manufacturers instructions and separated on a 0.8% agarose gel (in 1x Tris acetic acid buffer (TAE)) by electrophoresis at 30V for up to 48h. Lambda Mix Marker 19 (Fermentas) and bench top 1kb ladder (Promega) were used as size markers.

DNA preparation and linearization for pronuclear injection

BAC DNA was extracted from 1L E.coli culture in LB broth containing 15µg/mL Chlor using the Phase prep BAC DNA isolation kit (Sigma). After purification, DNA was air-dried and resuspended in injection buffer (10mM Tris pH7.5, 100mM NaCl, 0.1mM EDTA, 30µM spermine, 70µM spermidine) and stored at 4°C. 80µg of BAC DNA was linearized using MreI (NEB) at 37°C in a total volume of 500µL. The enzyme was heat inactivated at 65°C, and Heat inactivate enzyme 65°C 20min. Keep at 4°C, 400 µL of sterile filtered injection buffer and 20uL 6x DNA loading dye (Fermentas) added.

Sepharose column and pulse field electrophoresis

A sepharose column was created using a 5mL serological pipette (Sepharose CL-4B; Bioconcept). Therefore 11mL of sepharose was mixed with 5mL of injection buffer (10mM Tris pH7.5, 100mM NaCl, 0.1mM EDTA, 30µM spermine, 70µM spermidine), degassed and applied onto the column. The column was equilibrated with 30mL injection buffer, then the heat-inactivated linearized BAC DNA in injection buffer containing DNA loading dye applied onto the column and 22 fractions of a volume of 500µL eluted. DNA concentration and purity determined (nanodrop; Thermo scientific) and fractions run and separated by pulsed field electrophoresis in a 1.1% pulse field agarose (SIGMA) gel 0.5x Tris Borat EDTA buffer (TBE). As a molecular weight marker MidRange II PFG marker (New England Bioslabs) was used. Gel was poststained in EtBr containing 0.5xTBE buffer.

Pronuclear injection

B6D2F1 female mice were hormonally superovulated with 5 IU of Pregnant Mares Serum Gonadotropin and 5 IU of Human Chorion Gonadotropin i.p.. The

superovulated females were with B6D2F1 hybrid males. The next morning, fertilized one-cell eggs were scratched from the oviduct into M2 medium (Sigma). Cumulus cells were removed from the eggs with hyaluronidase (300 units/ml, Sigma); then the eggs were washed free of debris and enzyme. For injection, the eggs were transferred to a microinjection chamber in M2 medium and overlaid with oil. Eggs were sequentially held in place by a blunt pipet (outside diameter, 100 um) while the tip of the injector pipet was inserted through the zona pellucida and vitellus and into one of the pronuclei. The DNA solution (1-2ng/ul in injection buffer) in the injector pipette was slowly discharged into the pronucleous until a 30% distension of the pronucleus was observed. After injection the eggs were transferred M16 medium (Sigma) and cultured at 37°C for few hours, damaged cells were sorted out and the remaining ones transferred into the oviducts of pseudopregnant, random-bred CD1 mice.

Primers used for verification of BAC clones

BAC backbone PCRs

Primer BAC backbone/insert start forward: 5’ aaggagctgactgggttgaa 3’

Primer BAC backbone/insert start reverse: 5’ ctgtctgcaccctccttctc 3’

Primer BAC backbone/insert end forward: 5’ tggcacttcactctttgacg 3’

Primer BAC backbone/insert end reverse: 5’ cgatcctcccgaattgacta 3’

Mfge8 exon 1 locus PCR

Mfge8 Exon 1 forward: 5’ CACTTCAGCCCTCCCTCTTC 3’

Mfge8 Exon 1 reverse: 5’ CACGTTCGCCTCTCCTACC 3’

NeoR PCR

NeoR forward: 5’ gatcggccattgaacaagat 3’

NeoR reverse: 5’ gctccaatccttccattcaa 3’

Insertion start PCR (Mfge8 Exon1 to GOI STOP codon) forward primer in combination with GOI specific reverse primers

Mfge8 Exon 1 forward: 5’ CACTTCAGCCCTCCCTCTTC 3’

Cre insert reverse: 5’ GACCGCGCGCCTGAAGATAT 3’

CreERT2, tdTomato, Prnp, Lta: reverse: 5’ AGGCTAGAACTAGTGGATCT 3’

Insertion end PCR (second FRT to Intron 1)

FRT2 insert fwd: 5' GCTGAAGAGCTTGGCGGCGAAT 3'
Mfge8 Exon1 reverse 5' CACGTTCGCCTCTCCTACC 3'

Southern blot analysis

For the generation of the probe, PCRs using phusion polymerase were performed with following primer pairs

5' Probe

Primer forward: 5' gctcagcaaatgctttaccc 3'

Primer reverse: 5' agcatgctggagaccctaga 3'

3' Probe

Primer forward: 5' cttgtcctacgcctttcagc 3'

Primer reverse: 5' tgtcaagcattgcagagtcc 3'

PCR products were purified over gel and via GFX colum (GE healthcare).

1µg of BAC DNA was digested with AfeI (NEB), DNA separated over 0.8% agarose gel by electoporesis. Gel was rinsed in double distilled water (ddH_20), incubated in 0.2M HCl, washed in ddH_20, transferred into denaturing buffer (0.5 M NaOH), and neutralized in 0.5M Tris-HCl pH7.4 and transferred onto Hybond N+ membrane (GE healthcare) by capillary transport in 20xSSC buffer over night. DNA transferred onto nitrocellulose membrane is crosslinked by exposure to UV. The nitrocellulose blot was preincubated in hybridization solution for 2h 68°C (QuickHyb hybridyzation solution; Stratagene). The radioactively labelled probe was generated according to manufacturer's instructions, using previously purified PCR fragments for 5'/3'probe and radioactive α^{32}dCTP 3000Ci/mmol (Stratagene KIT Prime-It II random primer labelling kit). Probe was purified using GFX column and eluted in 50µL TE buffer pH8, 100µL salmon sperm DNA 10mg/mL (Stratagene) added, and denatured 2min 95°C and resuspended in 1mL hybridization solution, which was added to rotating flak containing 60mL hybridization solution and the Southern blot and incubated for 4h at 68°C. Membrane was washed in different concentrations of SSC buffer 2xSSC

0.1% SDS and 0.2xSSC 0.1% SDS at 68°C and the phosphoimager screen exposed for 24h to the membrane, for detection Fuji phosphoimager was used.

References

Alimzhanov, M. B., D. V. Kuprash, M. H. Kosco-Vilbois, A. Luz, R. L. Turetskaya, A. Tarakhovsky, K. Rajewsky, S. A. Nedospasov and K. Pfeffer (1997). "Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice." Proc Natl Acad Sci U S A **94**(17): 9302-7.

Aloisi, F. and R. Pujol-Borrell (2006). "Lymphoid neogenesis in chronic inflammatory diseases." Nat Rev Immunol **6**(3): 205-17.

Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Forster, J. D. Sedgwick, J. L. Browning, M. Lipp and J. G. Cyster (2000). "A chemokine-driven positive feedback loop organizes lymphoid follicles." Nature **406**(6793): 309-14.

Armulik, A., A. Abramsson and C. Betsholtz (2005). "Endothelial/pericyte interactions." Circ Res **97**(6): 512-23.

Balogh, P., Y. Aydar, J. G. Tew and A. K. Szakal (2001). "Ontogeny of the follicular dendritic cell phenotype and function in the postnatal murine spleen." Cell Immunol **214**(1): 45-53.

Baumann, I., W. Kolowos, R. E. Voll, B. Manger, U. Gaipl, W. L. Neuhuber, T. Kirchner, J. R. Kalden and M. Herrmann (2002). "Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus." Arthritis Rheum **46**(1): 191-201.

Blankenberg, F. G., P. D. Katsikis, J. F. Tait, R. E. Davis, L. Naumovski, K. Ohtsuki, S. Kopiwoda, M. J. Abrams, M. Darkes, R. C. Robbins, H. T. Maecker and H. W. Strauss (1998). "In vivo detection and imaging of phosphatidylserine expression during programmed cell death." Proc Natl Acad Sci U S A **95**(11): 6349-54.

Bolton, D. C., M. P. McKinley and S. B. Prusiner (1982). "Identification of a protein that purifies with the scrapie prion." Science **218**(4579): 1309-11.

Browning, J. L., I. Dougas, A. Ngam-ek, P. R. Bourdon, B. N. Ehrenfels, K. Miatkowski, M. Zafari, A. M. Yampaglia, P. Lawton, W. Meier and et al. (1995). "Characterization of surface lymphotoxin forms. Use of specific monoclonal antibodies and soluble receptors." J Immunol **154**(1): 33-46.

Büeler, H. R., A. Aguzzi, A. Sailer, R. A. Greiner, P. Autenried, M. Aguet and C. Weissmann (1993). "Mice devoid of PrP are resistant to scrapie." Cell **73**(7): 1339-47.

Burton, G. F., D. H. Conrad, A. K. Szakal and J. G. Tew (1993). "Follicular dendritic cells and B cell costimulation." J Immunol **150**(1): 31-8.

Campagnoli, C., I. A. Roberts, S. Kumar, P. R. Bennett, I. Bellantuono and N. M. Fisk (2001). "Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow." Blood **98**(8): 2396-402.

Cao, X., E. W. Shores, J. Hu-Li, M. R. Anver, B. L. Kelsall, S. M. Russell, J. Drago, M. Noguchi, A. Grinberg, E. T. Bloom and et al. (1995). "Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain." Immunity **2**(3): 223-38.

Cavert, W., D. W. Notermans, K. Staskus, S. W. Wietgrefe, M. Zupancic, K. Gebhard, K. Henry, Z. Q. Zhang, R. Mills, H. McDade, C. M. Schuwirth, J. Goudsmit, S. A. Danner and A. T. Haase (1997). "Kinetics of response in

lymphoid tissues to antiretroviral therapy of HIV-1 infection." Science **276**(5314): 960-4.

Cerny, A., R. M. Zinkernagel and P. Groscurth (1988). "Development of follicular dendritic cells in lymph nodes of B-cell-depleted mice." Cell Tissue Res **254**(2): 449-54.

Chaplin, D. D. and Y. Fu (1998). "Cytokine regulation of secondary lymphoid organ development." Curr Opin Immunol **10**(3): 289-97.

Chen, L. L., A. M. Frank, J. C. Adams and R. M. Steinman (1978). "Distribution of horseradish peroxidase (HRP)-anti-HRP immune complexes in mouse spleen with special reference to follicular dendritic cells." J Cell Biol **79**(1): 184-99.

Chin, R. K., J. C. Lo, O. Kim, S. E. Blink, P. A. Christiansen, P. Peterson, Y. Wang, C. Ware and Y. X. Fu (2003). "Lymphotoxin pathway directs thymic Aire expression." Nat Immunol **4**(11): 1121-7.

Cinamon, G., M. A. Zachariah, O. M. Lam, F. W. Foss, Jr. and J. G. Cyster (2008). "Follicular shuttling of marginal zone B cells facilitates antigen transport." Nat Immunol **9**(1): 54-62.

Cong, W., S. D. Finkelstein and M. Wu (2001). "[Relationship between genetic alterations and clinicopathological features in intrahepatic cholangiocarcinoma]." Zhonghua Bing Li Xue Za Zhi **30**(3): 183-7.

Cortes-Hernandez, J., L. Fossati-Jimack, A. Carugati, P. K. Potter, M. J. Walport, H. T. Cook and M. Botto (2002). "Murine glomerular mesangial cell uptake of apoptotic cells is inefficient and involves serum-mediated but complement-independent mechanisms." Clin Exp Immunol **130**(3): 459-66.

Cox, M. M. (1983). "The FLP protein of the yeast 2-microns plasmid: expression of a eukaryotic genetic recombination system in Escherichia coli." Proc Natl Acad Sci U S A **80**(14): 4223-7.

Cupedo, T., W. Jansen, G. Kraal and R. E. Mebius (2004). "Induction of secondary and tertiary lymphoid structures in the skin." Immunity **21**(5): 655-67.

Cupedo, T., G. Kraal and R. E. Mebius (2002). "The role of CD45+CD4+CD3- cells in lymphoid organ development." Immunol Rev **189**: 41-50.

Cyster, J. G., K. M. Ansel, K. Reif, E. H. Ekland, P. L. Hyman, H. L. Tang, S. A. Luther and V. N. Ngo (2000). "Follicular stromal cells and lymphocyte homing to follicles." Immunol Rev **176**: 181-93.

De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shornick, J. Strauss-Schoenberger and et al. (1994). "Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin." Science **264**(5159): 703-7.

Dellavalle, A., M. Sampaolesi, R. Tonlorenzi, E. Tagliafico, B. Sacchetti, L. Perani, A. Innocenzi, B. G. Galvez, G. Messina, R. Morosetti, S. Li, M. Belicchi, G. Peretti, J. S. Chamberlain, W. E. Wright, Y. Torrente, S. Ferrari, P. Bianco and G. Cossu (2007). "Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells." Nat Cell Biol **9**(3): 255-67.

Denning, K. L., P. R. Olson, R. H. Maley, Jr., V. R. Flati, J. L. Myers and J. F. Silverman (2009). "Primary pulmonary follicular dendritic cell neoplasm: a case report and review of the literature." Arch Pathol Lab Med **133**(4): 643-7.

DiSanto, J. P., W. Muller, D. Guy-Grand, A. Fischer and K. Rajewsky (1995). "Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain." Proc Natl Acad Sci U S A **92**(2): 377-81.

Eberl, G. and D. R. Littman (2003). "The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches." Immunol Rev **195**: 81-90.

Eberl, G., S. Marmon, M. J. Sunshine, P. D. Rennert, Y. Choi and D. R. Littman (2004). "An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells." Nat Immunol **5**(1): 64-73.

Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton and P. M. Henson (1992). "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages." J Immunol **148**(7): 2207-16.

Feil, R., J. Wagner, D. Metzger and P. Chambon (1997). "Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains." Biochem Biophys Res Commun **237**(3): 752-7.

Ferguson, A. R., M. E. Youd and R. B. Corley (2004). "Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells." Int Immunol **16**(10): 1411-22.

Flemming, W. (1885). "Studien über die Regeneration der Gewebe." Arch. Mikr. Anat. **24**: 50-91.

Forster, R., T. Emrich, E. Kremmer and M. Lipp (1994). "Expression of the G-protein--coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells." Blood **84**(3): 830-40.

Forster, R., A. E. Mattis, E. Kremmer, E. Wolf, G. Brem and M. Lipp (1996). "A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen." Cell **87**(6): 1037-47.

Fu, Y. X. and D. D. Chaplin (1999). "Development and maturation of secondary lymphoid tissues." Annu Rev Immunol.
17: 399-433.

Fukuyama, S., T. Hiroi, Y. Yokota, P. D. Rennert, M. Yanagita, N. Kinoshita, S. Terawaki, T. Shikina, M. Yamamoto, Y. Kurono and H. Kiyono (2002). "Initiation of NALT organogenesis is independent of the IL-7R, LTbetaR, and NIK signaling pathways but requires the Id2 gene and CD3(-)CD4(+)CD45(+) cells." Immunity **17**(1): 31-40.

Futterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois and K. Pfeffer (1998). "The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues." Immunity **9**(1): 59-70.

Gaipl, U. S., S. Franz, R. E. Voll, A. Sheriff, J. R. Kalden and M. Herrmann (2004). "Defects in the disposal of dying cells lead to autoimmunity." Curr Rheumatol Rep **6**(6): 401-7.

Gaipl, U. S., R. E. Voll, A. Sheriff, S. Franz, J. R. Kalden and M. Herrmann (2005). "Impaired clearance of dying cells in systemic lupus erythematosus." Autoimmun Rev **4**(4): 189-94.

Glatzel, M., F. L. Heppner, K. M. Albers and A. Aguzzi (2001). "Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion." Neuron **31**(1): 25-34.

Goldman, J. P., M. P. Blundell, L. Lopes, C. Kinnon, J. P. Di Santo and A. J. Thrasher (1998). "Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain." Br J Haematol **103**(2): 335-42.

Gray, D. (1988). "Recruitment of virgin B cells into an immune response is restricted to activation outside lymphoid follicles." Immunology **65**(1): 73-9.

Gu, H., J. D. Marth, P. C. Orban, H. Mossmann and K. Rajewsky (1994). "Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting." Science **265**(5168): 103-6.

Gu, H., Y. R. Zou and K. Rajewsky (1993). "Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting." Cell **73**(6): 1155-64.

Gunn, M. D., V. N. Ngo, K. M. Ansel, E. H. Ekland, J. G. Cyster and L. T. Williams (1998). "A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1." Nature **391**(6669): 799-803.

Guzman-Rojas, L., J. C. Sims-Mourtada, R. Rangel and H. Martinez-Valdez (2002). "Life and death within germinal centres: a double-edged sword." Immunology **107**(2): 167-75.

Hanayama, R., M. Tanaka, K. Miwa, A. Shinohara, A. Iwamatsu and S. Nagata (2002). "Identification of a factor that links apoptotic cells to phagocytes." Nature **417**(6885): 182-7.

Hanayama, R., M. Tanaka, K. Miyasaka, K. Aozasa, M. Koike, Y. Uchiyama and S. Nagata (2004). "Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice." Science **304**(5674): 1147-50.

Hardy, R. R. and K. Hayakawa (2001). "B cell development pathways." Annu Rev Immunol **19**: 595-621.

He, Y. W. and T. R. Malek (1996). "Interleukin-7 receptor alpha is essential for the development of gamma delta + T cells, but not natural killer cells." J Exp Med **184**(1): 289-93.

Heath, S. L., J. G. Tew, J. G. Tew, A. K. Szakal and G. F. Burton (1995). "Follicular dendritic cells and human immunodeficiency virus infectivity." Nature **377**(6551): 740-4.

Heggebo, R., C. M. Press, G. Gunnes, L. Gonzalez and M. Jeffrey (2002). "Distribution and accumulation of PrP in gut-associated and peripheral lymphoid tissue of scrapie-affected Suffolk sheep." J Gen Virol **83**(Pt 2): 479-89.

Heikenwalder, M., Federau C., von Boehmer, L., Schwarz, P., Wagner, M., Zeller, N., Haybaeck, J., Prinz, M., Becher, B. and Aguzzi A. (2007). "Germinal center B cells are dispensable in prion transport and neuroinvasion." J Neuroimmunol.

Heikenwalder, M., N. Zeller, H. Seeger, M. Prinz, P. C. Klohn, P. Schwarz, N. H. Ruddle, C. Weissmann and A. Aguzzi (2005). "Chronic lymphocytic inflammation specifies the organ tropism of prions." Science **307**(5712): 1107-10.

Heusermann, U., K. H. Zurborn, L. Schroeder and H. J. Stutte (1980). "The origin of the dendritic reticulum cell. An experimental enzyme-histochemical and electron microscopic study on the rabbit spleen." Cell Tissue Res **209**(2): 279-94.

Hjelmstrom, P. (2001). "Lymphoid neogenesis: de novo formation of lymphoid tissue in chronic inflammation through expression of homing chemokines." J Leukoc Biol **69**(3): 331-9.

Honda, K., H. Nakano, H. Yoshida, S. Nishikawa, P. Rennert, K. Ikuta, M. Tamechika, K. Yamaguchi, T. Fukumoto, T. Chiba and S. I. Nishikawa

(2001). "Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis." J Exp Med **193**(5): 621-30.

Hu, C. Y., C. S. Wu, H. F. Tsai, S. K. Chang, W. I. Tsai and P. N. Hsu (2009). "Genetic polymorphism in milk fat globule-EGF factor 8 (MFG-E8) is associated with systemic lupus erythematosus in human." Lupus **18**(8): 676-81.

Huber, C., C. Thielen, H. Seeger, P. Schwarz, F. Montrasio, M. R. Wilson, E. Heinen, Y. X. Fu, G. Miele and A. Aguzzi (2005). "Lymphotoxin-beta receptor-dependent genes in lymph node and follicular dendritic cell transcriptomes." J Immunol **174**(9): 5526-36.

Humphrey, J. H., D. Grennan and V. Sundaram (1984). "The origin of follicular dendritic cells in the mouse and the mechanism of trapping of immune complexes on them." Eur J Immunol **14**(9): 859-64.

Imal, Y. and M. Yamakawa (1996). "Morphology, function and pathology of follicular dendritic cells." Pathol Int **46**(11): 807-33.

Imazeki, N., A. Senoo and Y. Fuse (1992). "Is the follicular dendritic cell a primarily stationary cell?" Immunology **76**(3): 508-10.

Imazeki, N., A. Takeuchi, A. Senoo and Y. Fuse (1994). "New monoclonal antibodies directed against mouse follicular dendritic cells." J Histochem Cytochem **42**(3): 329-35.

Jeffrey, M., G. McGovern, C. M. Goodsir, K. L. Brown and M. E. Bruce (2000). "Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immuno-electron microscopy." J Pathol **191**(3): 323-32.

Johansson, T., I. Broll, T. Frenz, S. Hemmers, B. Becher, H. U. Zeilhofer and T. Buch "Building a zoo of mice for genetic analyses: A comprehensive protocol for the rapid generation of BAC transgenic mice." Genesis.

Kamperdijk, E. W., E. M. Raaymakers, J. H. de Leeuw and E. C. Hoefsmit (1978). "Lymph node macrophages and reticulum cells in the immune response. I. The primary response to paratyphoid vaccine." Cell Tissue Res **192**(1): 1-23.

Kapasi, Z. F., D. Qin, W. G. Kerr, M. H. Kosco-Vilbois, L. D. Shultz, J. G. Tew and A. K. Szakal (1998). "Follicular dendritic cell (FDC) precursors in primary lymphoid tissues." J Immunol **160**(3): 1078-84.

Kim, M. Y., G. Anderson, A. White, E. Jenkinson, W. Arlt, I. L. Martensson, L. Erlandsson and P. J. Lane (2005). "OX40 ligand and CD30 ligand are expressed on adult but not neonatal CD4+CD3- inducer cells: evidence that IL-7 signals regulate CD30 ligand but not OX40 ligand expression." J Immunol **174**(11): 6686-91.

Kitamoto, T., T. Muramoto, S. Mohri, K. Doh ura and J. Tateishi (1991). "Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease." J Virol **65**: 6292-5.

Kitamura, D., J. Roes, R. Kuhn and K. Rajewsky (1991). "A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene." Nature **350**(6317): 423-6.

Klaus, G. G. and J. H. Humphrey (1977). "The generation of memory cells. I. The role of C3 in the generation of B memory cells." Immunology **33**(1): 31-40.

Klein, M. A., R. Frigg, A. J. Raeber, E. Flechsig, I. Hegyi, R. M. Zinkernagel, C. Weissmann and A. Aguzzi (1998). "PrP expression in B lymphocytes is not required for prion neuroinvasion." Nat Med **4**(12): 1429-33.

Klein, M. A., P. S. Kaeser, P. Schwarz, H. Weyd, I. Xenarios, R. M. Zinkernagel, M. C. Carroll, J. S. Verbeek, M. Botto, M. J. Walport, H. Molina, U. Kalinke, H. Acha-Orbea and A. Aguzzi (2001). "Complement facilitates early prion pathogenesis." Nat Med **7**(4): 488-92.

Koni, P. A., R. Sacca, P. Lawton, J. L. Browning, N. H. Ruddle and R. A. Flavell (1997). "Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice." Immunity **6**(4): 491-500.

Koopman, G., H. K. Parmentier, H. J. Schuurman, W. Newman, C. J. Meijer and S. T. Pals (1991). "Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways." J Exp Med **173**(6): 1297-304.

Koopman, G., C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals and M. H. van Oers (1994). "Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis." Blood **84**(5): 1415-20.

Kosco-Vilbois, M. H. (2003). "Are follicular dendritic cells really good for nothing?" Nat Rev Immunol **3**(9): 764-9.

Kosco, M. H., E. Pflugfelder and D. Gray (1992). "Follicular dendritic cell-dependent adhesion and proliferation of B cells in vitro." J. Immunol. **148**(8): 2331-2339.

Kranich, J., N. J. Krautler, E. Heinen, M. Polymenidou, C. Bridel, A. Schildknecht, C. Huber, M. H. Kosco-Vilbois, R. Zinkernagel, G. Miele and A. Aguzzi (2008). "Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8." J Exp Med **205**(6): 1293-302.

Kuppers, R. (2003). "B cells under influence: transformation of B cells by Epstein-Barr virus." Nat Rev Immunol **3**(10): 801-12.

Kurebayashi, S., E. Ueda, M. Sakaue, D. D. Patel, A. Medvedev, F. Zhang and A. M. Jetten (2000). "Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis." Proc Natl Acad Sci U S A **97**(18): 10132-7.

Lakso, M., B. Sauer, B. Mosinger, Jr., E. J. Lee, R. W. Manning, S. H. Yu, K. L. Mulder and H. Westphal (1992). "Targeted oncogene activation by site-specific recombination in transgenic mice." Proc Natl Acad Sci U S A **89**(14): 6232-6.

Le Hir, M., H. Bluethmann, M. H. Kosco-Vilbois, M. Muller, F. di Padova, M. Moore, B. Ryffel and H. P. Eugster (1995). "Tumor necrosis factor receptor-1 signaling is required for differentiation of follicular dendritic cells, germinal center formation, and full antibody responses." J Inflamm **47**(1-2): 76-80.

Le Hir, M., H. Bluethmann, M. H. Kosco-Vilbois, M. Muller, F. di Padova, M. Moore, B. Ryffel and H. P. Eugster (1996). "Differentiation of follicular dendritic cells and full antibody responses require tumor necrosis factor receptor-1 signaling." J Exp Med **183**(5): 2367-72.

Lin, S. L., T. Kisseleva, D. A. Brenner and J. S. Duffield (2008). "Pericytes and perivascular fibroblasts are the primary source of collagen-producing cells in obstructive fibrosis of the kidney." Am J Pathol **173**(6): 1617-27.

Litman, G. W., J. P. Cannon and L. J. Dishaw (2005). "Reconstructing immune phylogeny: new perspectives." Nat Rev Immunol **5**(11): 866-79.

Liu, Y. J. and J. Banchereau (1997). "Regulation of B-cell commitment to plasma cells or to memory B cells." Semin Immunol **9**(4): 235-40.

Liu, Y. J., J. Zhang, P. J. Lane, E. Y. Chan and I. C. MacLennan (1991). "Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens." Eur J Immunol **21**(12): 2951-62.

Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers and R. Carsetti (1999). "B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals." J Exp Med **190**(1): 75-89.

Lu, T. T. and J. G. Cyster (2002). "Integrin-mediated long-term B cell retention in the splenic marginal zone." Science **297**(5580): 409-12.

Ludewig, B., S. Ehl, U. Karrer, B. Odermatt, H. Hengartner and R. M. Zinkernagel (1998). "Dendritic cells efficiently induce protective antiviral immunity." J Virol **72**(5): 3812-8.

Luther, S. A., K. M. Ansel and J. G. Cyster (2003). "Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development." J Exp Med **197**(9): 1191-8.

Mabbott, N. A., M. E. Bruce, M. Botto, M. J. Walport and M. B. Pepys (2001). "Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie." Nat Med **7**(4): 485-7.

Mabbott, N. A., F. Mackay, F. Minns and M. E. Bruce (2000). "Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie." Nat Med **6**(7): 719-20.

Mabbott, N. A., G. McGovern, M. Jeffrey and M. E. Bruce (2002). "Temporary blockade of the tumor necrosis factor receptor signaling pathway impedes the spread of scrapie to the brain." J Virol **76**(10): 5131-9.

Mabbott, N. A., A. Williams, C. F. Farquhar, M. Pasparakis, G. Kollias and M. E. Bruce (2000). "Tumor necrosis factor alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie." J Virol **74**(7): 3338-44.

Mabbott, N. A., J. Young, I. McConnell and M. E. Bruce (2003). "Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility." Journal of Virology **77**(12): 6845-54.

Mackay, F. and J. L. Browning (1998). "Turning off follicular dendritic cells." Nature **395**(6697): 26-7.

Masuda, A., G. F. Burton, B. A. Fuchs, A. K. Szakal and J. G. Tew (1993). "Destruction of follicular dendritic cells in murine acquired immunodeficiency syndrome (MAIDS)." Adv Exp Med Biol **329**: 411-6.

Masuda, A., G. F. Burton, A. K. Szakal and J. G. Tew (1995). "Loss of follicular dendritic cells in murine-acquired immunodeficiency syndrome." Lab Invest **73**(4): 511-20.

Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-5.

McBride, P. A., P. Eikelenboom, G. Kraal, H. Fraser and M. E. Bruce (1992). "PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice." J Pathol **168**(4): 413-8.

Mitchell, J. and A. Abbot (1965). "Ultrastructure of the antigen-retaining reticulum of lymph node follicles as shown by high-resolution autoradiography." Nature **208**(5009): 500-2.

Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa and V. E. Papaioannou (1992). "RAG-1-deficient mice have no mature B and T lymphocytes." Cell **68**(5): 869-77.

Montrasio, F., R. Frigg, M. Glatzel, M. A. Klein, F. Mackay, A. Aguzzi and C. Weissmann (2000). "Impaired prion replication in spleens of mice lacking functional follicular dendritic cells." Science **288**(5469): 1257-9.

Neutra, M. R., N. J. Mantis and J. P. Kraehenbuhl (2001). "Collaboration of epithelial cells with organized mucosal lymphoid tissues." Nat Immunol **2**(11): 1004-9.

Ohbo, K., T. Suda, M. Hashiyama, A. Mantani, M. Ikebe, K. Miyakawa, M. Moriyama, M. Nakamura, M. Katsuki, K. Takahashi, K. Yamamura and K. Sugamura (1996). "Modulation of hematopoiesis in mice with a truncated mutant of the interleukin-2 receptor gamma chain." Blood **87**(3): 956-67.

Ohteki, T., S. Ho, H. Suzuki, T. W. Mak and P. S. Ohashi (1997). "Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development." J Immunol **159**(12): 5931-5.

Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler and A. S. Fauci (1993). "HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease." Nature **362**(6418): 355-8.

Papamichail, M., C. Gutierrez, P. Embling, P. Johnson, E. J. Holborow and M. B. Pepys (1975). "Complement dependence of localisation of aggregated IgG in germinal centres." Scand J Immunol **4**(4): 343-47.

Pasparakis, M., L. Alexopoulou, V. Episkopou and G. Kollias (1996). "Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response " J Exp Med **184**(4): 1397-411.

Pasparakis, M., S. Kousteni, J. Peschon and G. Kollias (2000). "Tumor necrosis factor and the p55TNF receptor are required for optimal development of the marginal sinus and for migration of follicular dendritic cell precursors into splenic follicles." Cell Immunol **201**(1): 33-41.

Phan, T. G., J. A. Green, E. E. Gray, Y. Xu and J. G. Cyster (2009). "Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation." Nat Immunol **10**(7): 786-93.

Picarella, D. E., A. Kratz, C. B. Li, N. H. Ruddle and R. A. Flavell (1992). "Insulitis in transgenic mice expressing tumor necrosis factor beta (lymphotoxin) in the pancreas." Proc Natl Acad Sci U S A **89**(21): 10036-40.

Prinz, M., M. Heikenwalder, T. Junt, P. Schwarz, M. Glatzel, F. L. Heppner, Y. X. Fu, M. Lipp and A. Aguzzi (2003). "Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion." Nature **425**(6961): 957-62.

Prinz, M., M. Heikenwalder, P. Schwarz, K. Takeda, S. Akira and A. Aguzzi (2003). "Prion pathogenesis in the absence of Toll-like receptor signalling." EMBO Rep **4**(2): 195-9.

Prinz, M., F. Montrasio, M. A. Klein, P. Schwarz, J. Priller, B. Odermatt, K. Pfeffer and A. Aguzzi (2002). "Lymph nodal prion replication and neuroinvasion in mice devoid of follicular dendritic cells." Proc Natl Acad Sci U S A **99**(2): 919-24.

Prusiner, S. B. (1982). "Novel proteinaceous infectious particles cause scrapie." Science **216**(4542): 136-44.

Prusiner, S. B. (1991). "Molecular biology of prion diseases." Science **252**(5012): 1515-22.

Prusiner, S. B., M. P. McKinley, K. A. Bowman, D. C. Bolton, P. E. Bendheim, D. F. Groth and G. G. Glenner (1983). "Scrapie prions aggregate to form amyloid-like birefringent rods." Cell **35**(2 Pt 1): 349-58.

Qin, D., J. Wu, K. A. Vora, J. V. Ravetch, A. K. Szakal, T. Manser and J. G. Tew (2000). "Fc gamma receptor IIB on follicular dendritic cells regulates the B cell recall response." J Immunol **164**(12): 6268-75.

Racz, P. (1988). "Molecular, biologic, immunohistochemical, and ultrastructural aspects of lymphatic spread of the human immunodeficiency virus." Lymphology **21**(1): 28-35.

Rennert, P. D., J. L. Browning, R. Mebius, F. Mackay and P. S. Hochman (1996). "Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs." J Exp Med **184**(5): 1999-2006.

Reya, T., S. J. Morrison, M. F. Clarke and I. L. Weissman (2001). "Stem cells, cancer, and cancer stem cells." Nature **414**(6859): 105-11.

Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz and H. Bluethmann (1993). "Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes." Nature **364**(6440): 798-802.

Roufosse, C. A., N. C. Direkze, W. R. Otto and N. A. Wright (2004). "Circulating mesenchymal stem cells." Int J Biochem Cell Biol **36**(4): 585-97.

Sabin, F. R. (1902). "On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig." Am. J. Anat **1**: 367-38.

Santiago, A., T. Mori, J. Satriano and D. Schlondorff (1991). "Regulation of Fc receptors for IgG on cultured rat mesangial cells." Kidney Int **39**(1): 87-94.

Santiago, A., J. Satriano, S. DeCandido, H. Holthofer, R. Schreiber, J. Unkeless and D. Schlondorff (1989). "A specific Fc gamma receptor on cultured rat mesangial cells." J Immunol **143**(8): 2575-82.

Schrager, L. K. and A. S. Fauci (1995). "Human immunodeficiency virus. Trapped but still dangerous." Nature **377**(6551): 680-1.

Sedor, J. R., S. W. Carey and S. N. Emancipator (1987). "Immune complexes bind to cultured rat glomerular mesangial cells to stimulate superoxide release. Evidence for an Fc receptor." J Immunol **138**(11): 3751-7.

Shaner, N. C., R. E. Campbell, P. A. Steinbach, B. N. Giepmans, A. E. Palmer and R. Y. Tsien (2004). "Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein." Nat Biotechnol **22**(12): 1567-72.

Shaner, N. C., M. Z. Lin, M. R. McKeown, P. A. Steinbach, K. L. Hazelwood, M. W. Davidson and R. Y. Tsien (2008). "Improving the photostability of bright monomeric orange and red fluorescent proteins." Nat Methods **5**(6): 545-51.

Silvestre, J. S., C. Thery, G. Hamard, J. Boddaert, B. Aguilar, A. Delcayre, C. Houbron, R. Tamarat, O. Blanc-Brude, S. Heeneman, M. Clergue, M. Duriez, R. Merval, B. Levy, A. Tedgui, S. Amigorena and Z. Mallat (2005). "Lactadherin promotes VEGF-dependent neovascularization." Nat Med **11**(5): 499-506.

Smelt, S. C., C. R. Engwerda, M. McCrossen and P. M. Kaye (1997). "Destruction of follicular dendritic cells during chronic visceral leishmaniasis." J Immunol **158**(8): 3813-21.

Smith, J. P., A. M. Lister, J. G. Tew and A. K. Szakal (1991). "Kinetics of the tingible body macrophage response in mouse germinal center development and its depression with age." Anat Rec **229**(4): 511-20.

Soriano, P. (1994). "Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice." Genes Dev **8**(16): 1888-96.

Stavnezer, J., J. E. Guikema and C. E. Schrader (2008). "Mechanism and regulation of class switch recombination." Annu Rev Immunol **26**: 261-92.

Strick-Marchand, H., G. X. Masse, M. C. Weiss and J. P. Di Santo (2008). "Lymphocytes support oval cell-dependent liver regeneration." J Immunol **181**(4): 2764-71.

Sun, Z., D. Unutmaz, Y. R. Zou, M. J. Sunshine, A. Pierani, S. Brenner-Morton, R. E. Mebius and D. R. Littman (2000). "Requirement for RORgamma in thymocyte survival and lymphoid organ development." Science **288**(5475): 2369-73.

Takatori, H., Y. Kanno, W. T. Watford, C. M. Tato, G. Weiss, Ivanov, II, D. R. Littman and J. J. O'Shea (2009). "Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22." J Exp Med **206**(1): 35-41.

Tang, W., D. Zeve, J. M. Suh, D. Bosnakovski, M. Kyba, R. E. Hammer, M. D. Tallquist and J. M. Graff (2008). "White fat progenitor cells reside in the adipose vasculature." Science **322**(5901): 583-6.

Tew, J. G., G. J. Thorbecke and R. M. Steinman (1982). "Dendritic cells in the immune response: characteristics and recommended nomenclature (A report from the Reticuloendothelial Society Committee on Nomenclature)." J Reticuloendothel Soc **31**(5): 371-80.

Thielen, C., N. Antoine, F. Melot, J. Y. Cesbron, E. Heinen and R. Tsunoda (2001). "Human FDC express PrPc in vivo and in vitro." Dev Immunol **8**(3-4): 259-66.

Tsunoda, R., M. Nakayama, E. Heinen, K. Miyake, K. Suzuki, N. Sugai and M. Kojima (1992). "Emperipolesis of lymphoid cells by human follicular dendritic cells in vitro." Virchows Arch B Cell Pathol Incl Mol Pathol **62**(2): 69-78.

van de Pavert, S. A., B. J. Olivier, G. Goverse, M. F. Vondenhoff, M. Greuter, P. Beke, K. Kusser, U. E. Hopken, M. Lipp, K. Niederreither, R. Blomhoff, K. Sitnik, W. W. Agace, T. D. Randall, W. J. de Jonge and R. E. Mebius (2009). "Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation." Nat Immunol.

van Eijk, M., J. P. Medema and C. de Groot (2001). "Cutting edge: cellular Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein protects germinal center B cells from apoptosis during germinal center reactions." J Immunol **166**(11): 6473-6.

Vondenhoff, M. F., M. Greuter, G. Goverse, D. Elewaut, P. Dewint, C. F. Ware, K. Hoorweg, G. Kraal and R. E. Mebius (2009). "LTbetaR signaling induces cytokine expression and up-regulates lymphangiogenic factors in lymph node anlagen." J Immunol **182**(9): 5439-45.

Vondenhoff, M. F., G. Kraal and R. E. Mebius (2007). "Lymphoid organogenesis in brief." Eur J Immunol **37 Suppl 1**: S46-52.

Walport, M. J., K. A. Davies and M. Botto (1998). "C1q and systemic lupus erythematosus." Immunobiology **199**(2): 265-85.

Ware, C. F. (2005). "Network communications: lymphotoxins, LIGHT, and TNF." Annu Rev Immunol **23**: 787-819.

Ware, C. F., P. D. Crowe, M. H. Grayson, M. J. Androlewicz and J. L. Browning (1992). "Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells." J Immunol **149**(12): 3881-8.

Weissmann, C. (2004). "The state of the prion." Nat Rev Microbiol **2**(11): 861-71.

Yamaguchi, H., J. Takagi, T. Miyamae, S. Yokota, T. Fujimoto, S. Nakamura, S. Ohshima, T. Naka and S. Nagata (2008). "Milk fat globule EGF factor 8 in the serum of human patients of systemic lupus erythematosus." J Leukoc Biol **83**(5): 1300-7.

Yang, X. W., P. Model and N. Heintz (1997). "Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome." Nat Biotechnol **15**(9): 859-65.

Yoshida, K., T. K. van den Berg and C. D. Dijkstra (1993). "Two functionally different follicular dendritic cells in secondary lymphoid follicles of mouse spleen, as revealed by CR1/2 and FcR gamma II-mediated immune-complex trapping." Immunology **80**(1): 34-9.

Yoshida, T. and K. Takaya (1989). "Follicular dendritic reticular cells in the germinal center of the rat lymph node as studied by immuno-electron microscopy." Arch Histol Cytol **52**(4): 327-35.

Yoshida, T. and K. Takaya (1991). "Appearance of the reticular cells which trap antigens in the rat lymph node in postnatal development." J Electron Microsc (Tokyo) **40**(6): 378-84.

Zandvoort, A. and W. Timens (2002). "The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens." Clin Exp Immunol **130**(1): 4-11.

Zasloff, M. (2002). "Antimicrobial peptides of multicellular organisms." Nature **415**(6870): 389-95.

Zhang, N., J. Guo and Y. W. He (2003). "Lymphocyte accumulation in the spleen of retinoic acid receptor-related orphan receptor gamma-deficient mice." J Immunol **171**(4): 1667-75.

Zhang, Y., F. Buchholz, J. P. Muyrers and A. F. Stewart (1998). "A new logic for DNA engineering using recombination in Escherichia coli." Nat Genet **20**(2): 123-8.

ACKNOWLEDGEMENTS

I want to thank Professor Adriano Aguzzi, my mentor, for giving me the opportunity to pursue my thesis at his institute, which enabled me to work on a broad range of exciting projects including my thesis work. Under his supervision I elaborated my scientific understanding and knowledge. I am grateful for all his support over the last years, his bright ideas and his drive.

I am very thankful to the thesis committee, Prof. Annette Oxenius, Prof. Lucas Pelkmans, Prof. Christian Münz and Prof. Alexander Bürkle, for all their support and help during my thesis.

I want to specially mention Jan Kranich, who has made major contributions to the work on the FDCs.

Petra Schwarz for all the help with histological sections, stainings, organization of and work with mice, her support and supply with kilograms of cookies.

I am grateful to Anna Maria Calella and Sei-ichi Yusa, for their supervision during the first years and all the help and discussion during the second half of my PhD.

Many thanks to Mathias Heikenwälder for evaluating the PhD thesis, all the discussions we had and his support in different experiments.

José B. de Oliveira Martins for discussions and help during all the years.

Cristobal Tostado, Caihong Zhu, and Li Bei for help in the cloning of the BAC transgenic mice.

Special thanks to following colleagues
Mirzet Delic, Li-Chun Infanger, Dimitri Goriounov and the BZL-team for mouse work and genotyping. Norbert Wey, André Wethmar, Silvia Behnke and the

‘Odermatt lab’ for technical support. Frank Baumann, Mario Nuvolone, Gino Miele, Jeppe Falsig, Frank Heppner, Claire Bridel, Magdalini Polymendiou and all the other members from the institute of neuropathology for discussions and support. Jacqueline Wiedler, Sarah Amzallag and Catherine Schindler for organizational work.

For collaborations:
I want to thank especially to Dr. Thorsten Buch and Dr. Pawel Pelczar for the collaborations on the generation of the BAC transgenic mice and supply with different mouse lines. Thorbjörn Johannson and Florian Mair for technical help.

Dr. Michelle Tallquist for the Pdgfrb-Cre x R26RlacZ spleens. Dr. Ralf Adams supplied me with Pdgfrb-Cre mice. Dr. Karl Lang and Prof. Zinkernagel for the μMT^{D} and $Tnfa^{-/-}$ mice. Jeffrey Browning for LTβR-Ig fusion proteins and agonistic anti-LTβR antibodies.

I am furthermore very grateful to my father Berhard Kräutler for scientific input and my mother Rita Kräutler for proof reading.

.

CURRICULUM VITAE

Nike KRÄUTLER

Date of Birth: January 17th, 1981

Place of birth: Zurich, Switzerland

Nationalities: Austrian, German

Education and Training

6/2005-present **PhD at the Institute of Neuropathology**
Prof. Adriano Aguzzi, University Hospital Zurich, Switzerland
Title: The origin of follicular dendritic cells

12/2004-5/2005 **Scientific co-worker at the Institute of Neuropathology**
Prof. Adriano Aguzzi, University Hospital Zurich, Switzerland

10/1999-4/2004 **Studies in biology at the Swiss Federal Institute of Technology (ETH) and Max-Planck Institute (MPI) for Immunology**
ETH, Zürich, Switzerland **,** MPI Freiburg, Germany

Studies in biology

- 10/1999-10/2001: Undergraduate degree

Specialization in neurosciences

- 10/2002-1/2003: Internship at the MPI
- 2/2003-8/2003: Diploma thesis at the Institute of Brain Research, ETH Zürich, in collaboration with the MPI for Immunology
- Title: Jagged1: a modulator of midbrain-hindbrain formation and cerebellar development
- Graduation (Dipl. Natw.) at the ETH 4/2004 corresponding to a Master of Science

11/1991-7/1999 **High School: Akademisches Gymnasium Innsbruck, Austria.** Matura, qualification for university studies

1987-1991 **Elementary School**

1987-1990 Primarschule Stäfa, Switzerland

1990-1991 Primarschule Fluntern Zurich, Switzerland

PRESENTATIONS

Oral presentation at the 16th Germinal Center Conference 2009, Frankfurt, Germany. Title: **Mfge8 – more than just a marker for FDCs**

Oral presentation at the Alpine Meeting "Molecular basis of disease" 2009, Schwarzenberg, Switzerland. Title: **Mfge8 – more than just a marker for FDCs**

Oral presentation at the NCCR Minisymposium on Transgenesis 2009, Zurich, Switzerland. Title: **Mfge8 BACs – tools to target questions related prion diseases and the development of secondary/tertiary lymphoid organs**

Oral presentation at the Alpine Meeting "Molecular basis of disease" 2008, Klöntal, Switzerland. Title: **Follicular dendritic cells rising**

Poster presentation at the 16th Germinal Center Conference 2009, Frankfurt, Germany. Title: **Putative precursors of follicular dendritic cells in the absence of B Cells and LTbR signalling**

Poster presentation at the World Immune Regulation Meeting III, 2009, Davos, Switzerland. Title: **Dissecting the development of follicular dendritic cells**

Poster presentation at the PRION meeting 2007 , Edinburgh, Scotland
Title: **The prion protein interactome during health and disease – a proteomic analysis**

PUBLICATIONS

Engulfment of cerebral apoptotic bodies controls the course of prion disease.
Krautler NJ*, Kranich J*, Jeppe Falsig, Boris Ballmer, Gregor Hutter, Petra Schwarz, Rita Moos, Christian Julius, Gino Miele, Adriano Aguzzi
Manuscript in review, * contributed equally

The follicular dendritic cell emerges from perivascular precursors.
Krautler NJ, Kranich J, Schwarz P, Heikenwalder M, Browning J, Tallquist M, Adams R, Delic M, Tostado C, Oliveira-Martins J, Aguzzi A
Manuscript in preparpation

Repetitive immunization enhances the susceptibility of mice to peripherally administered prions. Bremer J, Heikenwalder M, Haybaeck J, Tiberi C, **Krautler NJ**, Kurrer MO, Aguzzi A. PLoS One. 2009 Sep 25;4(9):e7160.

Bacterial colitis increases susceptibility to oral prion disease. Sigurdson CJ, Heikenwalder M, Manco G, Barthel M, Schwarz P, Stecher B, **Krautler NJ**, Hardt WD, Seifert B, MacPherson AJ, Corthesy I, Aguzzi A. J Infect Dis. 2009 Jan 15;199(2):243-52.

Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8. Kranich J, **Krautler NJ**, Heinen E, Polymenidou M, Bridel C, Schildknecht A, Huber C, Kosco-Vilbois MH, Zinkernagel R, Miele G, Aguzzi A. J Exp Med. 2008 Jun 9;205(6):1293-302.

Jagged1 ablation results in cerebellar granule cell migration defects and depletion of Bergmann glia. Weller M, **Krautler N**, Mantei N, Suter U, Taylor V. Dev Neurosci. 2006;28(1-2):70-80.